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Yu-Jie Hu
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ROLES OF PROTEIN ARGININE METHYLTRANSFERASE 7 AND JUMONJI DOMAIN-CONTAINING PROTEIN 6 IN ADIPOCYTE DIFFERENTIATION

A Dissertation Presented

By

Yu-Jie Hu

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

October 28th, 2015

Program in Cell Biology

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Program in Cell Biology

October 28th, 2015

Dedication

This thesis is dedicated to my family and friends for their support.

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I would like to thank my thesis advisor Dr. Tony Imbalzano for his mentoring throughout these years. His advice and encouragement were the most important support during my graduate research. I thank him for being so supportive and thoughtful to me. I wish someday I could be such a nice and warm person as he is.

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Abstract

Regulation of gene expression comprises a wide range of mechanisms that control the abundance of gene products in response to environmental and developmental changes. These biological processes can be modulated by post-translational modifications including arginine methylation. Among the enzymes that catalyze the methylation, protein arginine methyltransferase 7 (PRMT7) is known to modify histones to repress gene expression. Jumonji domain-containing protein 6 (JMJD6) is a putative arginine demethylase that potentially antagonize PRMT7. However, the biological significance of these enzymes is not well understood. This thesis summarizes the investigation of both PRMT7 and JMJD6 in cell culture models for adipocyte differentiation. The results suggest that PRMT7 is not required for the differentiation, whereas JMJD6 is necessary for the differentiation by promoting the expression of the lineage determining transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs). The underlying mechanisms by which JMJD6 regulate differentiation involve transcriptional and post-transcriptional control of gene expression. Unexpectedly, the adipogenic function of JMJD6 is independent of its enzymatic activity. Collectively, the present research reveals a novel role of JMJD6 in gene regulation during the differentiation of adipocytes.

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Figure Number	Publisher
All Chapter II figures	F1000Research
All Chapter III figures	Nucleic Acids Research

Preface

Parts of this dissertation appear in the following published work:

Hu, Y.J., Sif, S., Imbalzano, A.N. (2013) Prmt7 is dispensable in tissue culture models for adipogenic differentiation. *F1000Res*. 2:279.

Hu, Y.J., Belaghzal, H., Hsiao, W.Y., Qi, J., Bradner, J.E., Guertin, D.A, Sif, S., Imbalzano, A.N. (2015) Transcriptional and post-transcriptional control of adipocyte differentiation by Jumonji domain-containing protein 6. *Nucleic Acids Res*. 43(16): 7790-7804.

Other published work during graduate study that are not presented in this thesis:

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LeBlanc, S.E., Konda, S., Wu, Q., **Hu, Y.J**., Oslowski, C.M., Sif, S., Imbalzano, A.N. (2012) Protein arginine methyltransferase 5 (Prmt5) promotes gene expression of peroxisome proliferator-activated receptor γ2 (PPARγ2) and its target genes during adipogenesis. *Mol. Endocrinol.* 26(4): 583-97

Karkhanis, V., **Hu, Y.J**., Baiocchi, R.A., Imbalzano, A.N., Sif, S. (2011) Versatility of PRMT5-induced methylation in growth control and development. *Trends Biochem Sci*. 36(12): 633-41

Mallappa, C., **Hu, Y.J.**, Shamulailatpam, P., Tae, S., Sif, S., Imbalzano, A.N. (2010) The expression of myogenic microRNAs indirectly requires protein arginine methyltransferase (Prmt)5 but directly requires Prmt4. *Nucleic Acids Res*. 39(4):1243-55

In Chapter II, the pBABE-PRMT7 construct was provided by Dr. Saïd Sif.

In Chapter III, the mouse tissue extracts were provided by Wen-Yu Hsiao and Dr. David Guertin. JQ1 was provided by Dr. Jun Qi and Dr. James Bradner. The JMJD6 antisera was provided by Dr. Saïd Sif. The gene expression analysis in Figure 3-4D was done by Houda Belaghzal.

CHAPTER I

Introduction

1.1 Protein arginine methylation

Arginine has the most basic side-chain (pKa=12.48) among the twenty amino acids. At physiological pH, the guanidinium group of arginine side-chain is protonated and contains positive charges. This chemical property allows arginine to form multiple hydrogen bonds with electronegative molecules such as nucleic acids (1). In mammalian cells, arginine can be methylated to form ω-N^G-monomethylarginine (MMA), ω- N^G- N^G-asymmetric dimethylarginine (ADMA), and ω- N^G- N'^G-symmetric dimethylarginine (SDMA) (2-4) (**Figure I-1**). The addition of methyl groups on arginine residues increases hydrophobicity and reduces hydrogen bonding. These effects could change protein structure and affect protein-protein or protein-nucleotide interactions (1).

Arginine methylation is a common post-translational modification. Around 2 % of the arginine residues in rat liver nuclei and 1% of total arginine residues in human lung epithelial cells are methylated (5,6). Mass spectrometry (MS)-based proteomics either combined with immunopurification using methylation-specific antibodies or heavy methyl-stable isotope labeling by amino acids in cell culture (SILAC) method identified hundreds of arginine-methylated proteins in both human

and mouse cells (7-12). RNA processing factors and transcription regulators are predominant among the identified proteins, suggesting that arginine methylation may play significant roles in regulating gene expression at the steps of transcription and RNA processing in mammalian cells.

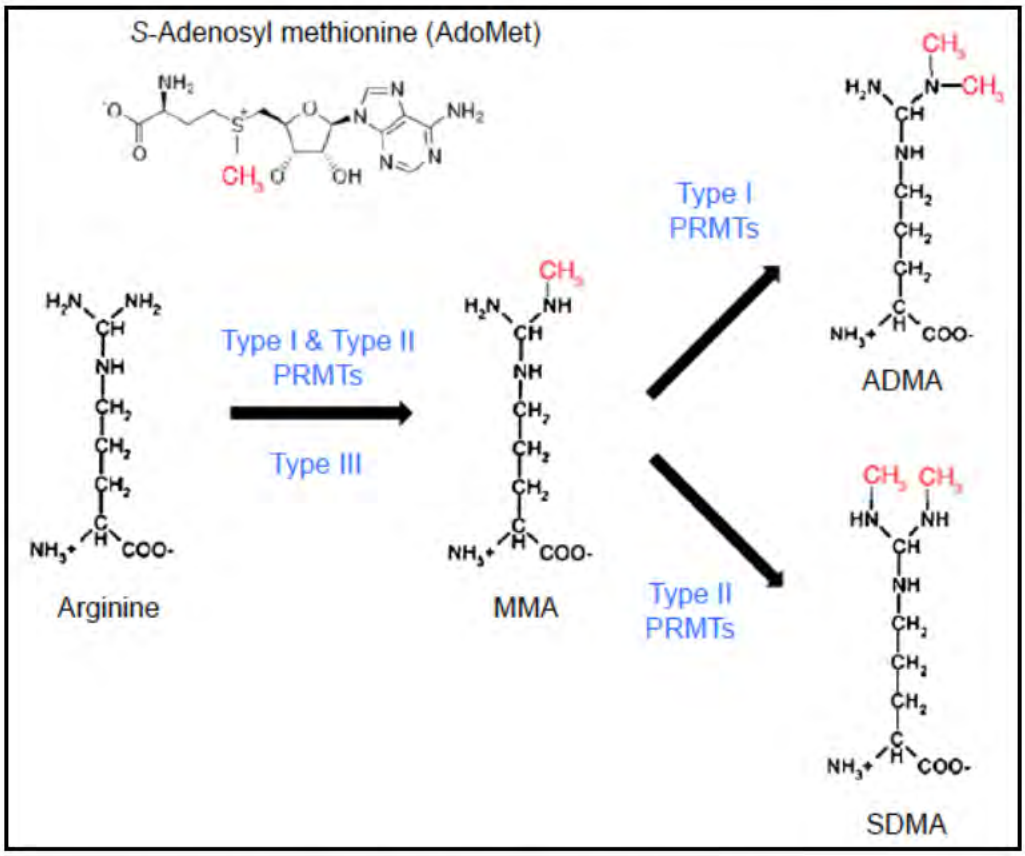

Figure I-1 Arginine methylation and methyltransferases.
Protein arginine methyltransferases (PRMTs) transfer one or two methyl groups from S-adenosyl-methionine (AdoMet) to the guanidinium group of arginine side-chain to give rise to ω-N^G-monomethylarginine (MMA), ω- N^G-N^G-asymmetric dimethylarginine (ADMA), and ω- N^G- N'^G-symmetric dimethylarginine (SDMA).

1.2 Protein arginine methyltransferases

Arginine methylation is catalyzed by protein arginine methyltransferases (PRMTs) (**Figure I-1**). PRMTs transfer one or two methyl groups from S-adenosyl-

methionine (AdoMet) to the guanidinium group of arginine side-chain and produce methylarginine and S-adenosylhomocysteine (AdoHcy). Nine PRMT genes have been identified in the mammalian genome (4). According to the methylation products, PRMTs are classified into three types (2): PRMT1, PRMT2, PRMT3, PRMT4, PRMT6 and PRMT8 are the type I PRMTs that produce ADMA (13-17). PRMT5 and PRMT9 are the type II PRMTs that deposit SDMA on substrates (18-21). PRMT7 is assigned as a type III PRMT that only catalyzes MMA (22-25). However, some evidence suggests that PRMT7 has a type II activity (26-33).

The PRMT family members share the characteristic catalytic domain composed of five conserved motifs named motif I, motif post I, motif II, motif III, and threonine-histidine-tryptophan (THW) loop (1,4,34) (**Figure I-2**). Motif I to motif III form a seven-stranded β sheet structure for AdoMet binding, whereas the THW loop helps substrate recognition by stabilizing the N-terminal helix of the enzyme. PRMT7 and PRMT9 differ from the other PRMTs, because they contain two putative catalytic domains. Both catalytic domains are essential for their enzymatic activities (21,22). Biochemical characterization showed that the N-terminal catalytic core of PRMT7 binds AdoMet, whereas the C-terminal catalytic core does not (22,35), suggesting that the C-terminal catalytic core only plays a structural role for the enzymatic function.

In addition to the catalytic domain, PRMTs contain a signature motif called double E loop (1,4). The double E loop is following motif II and comprises of two

glutamate residues separated by eight amino acids. Crystallographic and mutagenesis analyses demonstrated that the conserved glutamate residues form hydrogen bond interactions with the arginine side-chain and are critical for enzymatic activity (35-40). The other amino acids within the double E loop are crucial for substrate selection (25).

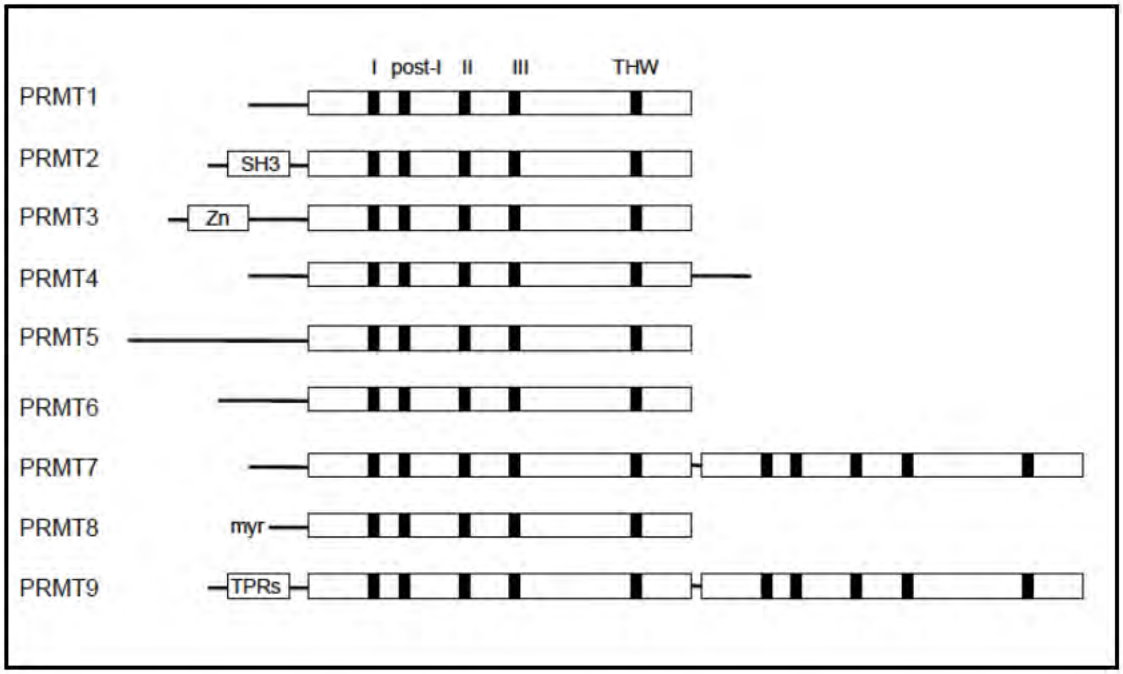

Figure I-2. Domains and motifs of PRMT family members.
PRMTs share the characteristic catalytic domain composed of five conserved motifs named motif I, motif post I, motif II, motif III, and THW loop. SH3: SH3 domain; Zn: zinc finger domain; myr: N-myristoylation; TPRs: tetratricopeptide repeats.

Additional protein domains in individual PRMTs regulate enzymatic activity, substrate recognition or cellular localization. For instance, PRMT2 has a SH3 domain at the N-terminus that mediates its interaction with other protein such as PRMT8 (41). The zinc finger domain at the N-terminus of PRMT3 interacts with its

substrate, the ribosomal protein S2 (42). PRMT8 contains a myristoylation motif at the N-terminus, which is required for its targeting to the plasma membrane (17). The N-terminal region of PRMT9 contains three tetratricopeptide repeats (TPR), which could form an α-helix pair structure that mediates protein-protein interactions (43).

1.3 Mouse models for PRMTs

To understand the physiological functions of PRMTs in mammals, a number of genetically engineered mouse models have been generated (**Table 1**). The PRMT1 (44,45), PRMT4 (46,47), PRMT5 (48), and PRMT7 (33) deficient mice showed a range of developmental defects, whereas the PRMT2 (49), PRMT3 (50) and PRMT6 (51) deficient mice are viable without gross developmental abnormalities. A tamoxifen-inducible ER-PRMT6 transgenic mouse line is the only gain-of-function model that has been reported (52).

PRMT1, PRMT4, PRMT5, and PRMT7 play significant and non-redundant roles in the cell growth, differentiation, or viability. Both PRMT1 hypomorphic and knockout embryos die shortly after implantation (44,45). PRMT5 knockout embryos die by E6.5 (48). The early embryonic lethality suggests that PRMT1 and PRMT5 are indispensable for proliferation and differentiation during early embryogenesis. PRMT4/CARM1 null mice die shortly after birth with smaller body size and defects in multiple tissues such as lung, T lymphocyte, adipose, muscle and chondrocyte (46,53-58). A mouse line with knock-in alleles of PRMT4 catalytic-inactive mutation showed the same phenotypes as the PRMT4 null mice,

indicating that the methyltransferase activity of PRMT4 is essential for its role *in vivo* (47). The PRMT7 knockout mice have no obvious developmental defects at birth, however, most animals died within 5-10 days (33). Moreover, the B-cell specific PRMT7 knockout mice showed impaired B cell development in spleen and defective humoral immune responses (33).

Table 1. Mouse models for PRMTs

PRMT / allele	Mutant phenotype	Refs
PRMT1 / hypomorphic	ES cells are viable. Embryos fail to develop beyond E8.5.	(44)
PRMT1 / conditional null	Embryos do not survive to E7.5. MEFs show genomic instability and cell death.	(45)
PRMT2 / null	Mice are viable. MEFs show early S-phase entry, resistant to apoptosis, and increased NF-κB activity.	(49,59)
PRMT3 / hypomorphic	Mice are viable. Embryos are smaller than the wild-type embryos at E13.5 and E18.5.	(50)
PRMT4 / conditional null	Neonatal lethality. Differentiation defects of T cells, adipocytes, chondrocytes, muscle cells, and pulmonary epithelial cells.	(46,53-58)
PRMT4 / enzyme-dead knock-in	The mutant mice phenocopy the PRMT4-null mice.	(47)
PRMT5 / null	Blastocysts appear normal at E3.5, but embryos die by E6.5. No PRMT5-null ES cells could be obtained from blastocysts.	(48)
PRMT6 / conditional null	Mice are viable. MEFs exhibited growth defects and cellular senescence.	(51)
PRMT6 / ER-PRMT6 knock-in	Mice have increased serum IL-6 levels. MEFs show enhanced NF-κB activity.	(52)
PRMT7 / conditional null	A conditional null allele. Most mutant mice die within 5-10 days after birth. B cell differentiation	(33)

	in the bone marrow of B-cell specific knockout mice appears normal, but late B-development in spleen and humoral responses are impaired.	

Cells derived from different PRMT mouse models have been used for studying cellular and molecular functions of these enzymes. For example, the tamoxifen-inducible PRMT1$^{fl/-}$ ER-Cre MEF line was used to determine the function of PRMT1 in cell viability and genome stability (45). The PRMT2 knockout MEFs have an increased NF-κB activity and are more resistant to apoptosis than the wild-type cells (49). The PRMT4 knockout MEFs showed deficiencies in estrogen receptor, c-fos, PPARγ, and NF-κB-mediated transcription (46,55,60,61). Introduction of muscle lineage transcription factors into PRMT4 knockout MEFs failed to induce muscle genes and miRNA expression (56,62). PRMT6 knockout MEFs are prone to cellular senescence due to the activation of p53 pathway (51).

Primary cells derived from PRMT mouse models were also used for substrates identification and antibody validation. For instance, a panel of MMA-, ADMA- and SDMA-specific antibodies was used to detect global arginine methylation in the PRMT1 knockout MEFs (63). This study not only showed that PRMT1 is the predominant enzyme responsible for ADMA modification, but also revealed an increase of MMA and SDMA levels when PRMT1 was depleted (63). The inducible PRMT5 knockout MEFs were used to quantify the SDMA levels that contributed by this enzyme (21). The results indicated that PRMT5 accounts for

the majority of SDMA in the MEFs, and the remaining type II activity might come from PRMT9 (21).

1.4 Regulation of gene expression by PRMTs

PRMTs participate in a wide range of mechanisms that control gene expression (1-4,64-68). PRMTs act as co-regulators for gene transcription and also modify histones and transcription regulators. In the subsequent step of gene expression, PRMTs methylate proteins involved in RNA processing and translation. These diverse functions of PRMTs in the regulation of gene expression are summarized below.

1.4.1 Histone arginine methylation and chromatin regulation

Histones are the basic proteins that help to package DNA into structure in the nucleus. They are well-identified substrates for PRMTs. Different PRMTs can methylate histones at distinct residues that associate with different transcriptional outcomes (**Table 2**).

Table 2. Histone arginine methylation and the effects on transcription

PRMT	Methylated Histone and Transcriptional Effects	Ref
PRMT1	H4R3 (active)	(69-71)
PRMT2	H3R8 (unknown)	(72)
PRMT4	H3R17 (active), H3R26 (active)	(73,74)
PRMT5	H3R8 (active/repressive); H4R3 (repressive); H2AR3 (repressive)	(48,56,75-79)

PRMT6	H3R2 (repressive); H2AR29 (repressive); H3R42 (active); H2AR3 (unknown); H4R3 (unknown)	(66,80-85)
PRMT7	H2AR3 (repressive), H4R3 (repressive), H3R2(active), H2BR29/31/33 (unknown)	(24,27,30-33)

The mechanistic connections between histone methylation by PRMTs and gene transcription has been suggested. Histone arginine methylation can recruit other epigenetic modifiers to establish an active or repressive chromatin environment for subsequent transcription events. For instance, PRMT1-mediated asymmetrical dimethylation on histone H4R3 facilitates subsequent histone acetylation, because the methylated histone H4R3 is better substrate for CBP/p300 acetyltransferase (69-71). PRMT5-mediated H4R3 symmetrical dimethylation recruit DNA methyltransferase 3α (DNMT3A) to silence the human γ-globin gene via DNA methylation at the CpG sites around the promoter (86). Methylation on histone H4R3 by PRMT7 inhibits mixed-lineage leukemia 4 (MLL4) -mediated H3K4 methylation, and leads to gene repression (87). In addition, methylation of H3K4 is prevented by PRMT6-mediated H3R2 methylation, which is supported by structural and biochemical studies (80-83).

Histone arginine methylation also regulates the chromatin binding of effector proteins that recognize the methylation state of arginine residues. For instance, the asymmetrical dimethylation on H4R3 and H3R17 is recognized by Tudor domain-containing protein 3 (TDRD3), a protein specifically binds

methylarginine peptides (88). Chromatin immunoprecipitation coupled with high-throughput DNA sequencing (ChIP-seq) analysis showed that TDRD3 is generally associated with transcription start sits (TSSs) (88), suggesting that it is an effector protein for active methylarginine marks deposited by PRMT1 and PRMT4. Methylation on histones can also prevent the chromatin binding of protein factors. For example, PRMT1 and PRMT5-mediated H4R3 dimethylation inhibits the chromatin binding of signal recognition particle subunits SRP68 and SRP72 (89). Knockdown of SRP68 causes changes in its target gene expression, suggesting a function of SRP68 in transcription (89). Thus, histone arginine methylation is a part of the histone code (90) that regulates chromatin function and transcription.

Several PRMTs collaborate with chromatin remodeling complexes to affect gene transcription. It is known that PRMT4, PRMT5 and PRMT7 are associated with the mammalian SWI/SNF complexes (31,76,91). The SWI/SNF complex is a multi-subunit protein complex that contains the ATPase Brahma (BRM) or Brahma-related gene 1(BRG1) for remodeling chromatin (92-95). PRMT4 and the SWI/SNF complex constitutes a nucleosomal methylation activator complex (NUMAC), where PRMT4 acquires the ability to methylate nucleosomal histones and also stimulates the catalytic activity of BRG1 (91). PRMT5 and PRMT7 methylate nucleosomes more efficiently when they are associated with the SWI/SNF complex (68,76). Moreover, the loss of PRMTs can lead to a reduced binding of the SWI/SNF complex at the target gene promoters and gene expression (56,76-79), indicating that PRMTs not only fine-tunes the activity of the

chromatin remodeling complexes but also assist the loading of the remodeling enzymes to affect gene transcription.

PRMT5 is also part of MBD2-NURD complexes (96,97), Ski co-repressor complexes (98) and Mediator complexes (99). When PRMT5 is associated with MBD2-NURD complex, it methylates histone H4R3 and MBD2 within the arginine/glycine-rich region at N-terminus (96). The methylation of MBD2 reduced its DNA binding affinity and the formation of MBD2-NURD complex at the target promoters (97). PRMT5 was co-purified with histone deacetylates 3 (HDAC3) in the Ski complexes (98). The binding of PRMT5 and HDAC3 at the *SMAD7* gene promoter correlates with the gene repression, suggesting that the collaboration of PRMT5 and HDAC3 establish a repressive chromatin environment for gene transcription. PRMT5 and its cofactor WDR77 (also called MEP50) are associated with the Mediator complexes to repress C/EBPβ target genes by deposition of methylated histone H4R3 and the subsequent DNA methylation (99). Therefore, it is clear that PRMT5 cooperates with the components of different chromatin complexes to regulate chromatin environment and transcription.

1.4.2 Methylation of non-histone proteins in transcriptional controls

PRMTs can modify signal transducers, transcription factors, co-regulators, and the transcription machinery to have effects on gene transcription (64). For instance, PRMT1 methylates estrogen receptor α (ERα) within the DNA binding domain upon estrogen stimulation in the MCF7 human breast cancer cell line

(100). This methylation facilitates the assembly of ERα with SRC and focal adhesion kinase (FAK) in the cytoplasm, leading to the activation of the AKT signaling pathway. PRMT1 also methylates FOXO1, a transcription factor involved in glucose metabolism and adipocyte differentiation (101). The methylation occurs in a consensus motif for AKT phosphorylation and prevents the phosphorylation by AKT. Consequently, FOXO1 is retained in the nucleus allowing gene expression. Other components of signal transduction pathways such as interferon receptor (102,103), JAK-STAT (104,105), TGFβ-SMAD (106), and Wnt (107) signaling pathways are also substrates for PRMT1. This evidence highlights the participation of PRMT1 in diverse biological processes by modifying signaling proteins and modulating the subsequent transcriptional responses.

PRMT4 and PRMT5 also methylate transcription factors in response to external stimuli. PRMT4 methylates C/EBPβ, a MAP kinase signal-sensitive transcription factor regulating proliferation, differentiation, immunity and tumorigenesis (108). The methylation is abolished by MAP kinase-mediated phosphorylation at a conserved site within the regulatory domain of C/EBPβ. The substitution of arginine with lysine prevents C/EBPβ from interacting with SWI/SNF and Mediator complexes. The cells harboring this mutation failed to activate gene expression during myeloid and adipogenic differentiation, suggesting this methylation site is crucial for C/EBPβ to activate transcription. Similarly, the transcription activity of p53 can be modulated by arginine methylation in response to DNA damage (109). Ectopic expression of PRMT5 could lead to p53-dependent

cell cycle arrest, whereas depletion of PRMT5 triggers apoptosis. Thus, the protein level and methylation status of p53 could alter cellular response to stress.

Transcription co-regulators are proteins that interact with transcription factors to either activate or repress gene transcription. The activity and assembly of co-regulator complexes can be modulated by arginine methylation. PRMT1 methylates peroxisome proliferator-activated receptor γ co-activator 1-α (PGC-1α) (110) and nuclear receptor-interacting protein 1 (NRIP1, also known as RIP140) (111). PGC-1α and RIP140 are co-activators for the genes involved in energy metabolism. PRMT1-mediated methylation within a glutamate-and arginine-rich region at the C-terminus promotes PGC-1α's co-activator activity. In contrast, PRMT1-mediated methylation triggers the dissociation of RIP140 from histone deacetylases and nuclear export of RIP140, suppressing its co-repressor activity.

PRMT4 methylates CBP and p300 co-activators to promote their interaction with cAMP- response element-binding protein (CREB) and the steroid receptor co-activators (SRCs) (112-114). In contrast, PRMT4 methylates the C-terminus of SRC-3, one of the SRC/p160 co-activator family members, leading to dissociation of PRMT4 and CBP from SRC-3 (115,116). Therefore, arginine methylation can be a regulatory signal for the assembly and disassembly of co-activator complexes.

The components of the transcription machinery are also targets of PRMTs. PRMT4 methylates the residue of arginine 1810 within the C-terminal domain of

RNA polymerase II (117). Substitution of arginine to alanine at the methylation site results in altered expression of a variety of small nuclear RNAs and small nucleolar RNAs. Moreover, the methylation occurs on the hyperphosphorylated form of RNA polymerase II, and phosphorylation at serine 2 and serine 5 inhibits the methylation. This evidence suggests a cross-regulation between methylation and phosphorylation on the C-terminal domain of RNA polymerase that contributes to the transcription of selected genes.

SPT5 is a transcriptional elongation factor that forms a heterodimer with SPT4 to positively or negatively regulate transcription elongation depending on the status of posttranslational modifications. The positive transcription elongation factor (P-TEFb) phosphorylates SPT5 into its stimulatory form (118,119). In contast, both PRMT1 and PRMT5 methylate SPT5, leading to a decreased association with RNA polymerase II and a repression of gene transcription (120).

1.4.3 RNA processing

RNA processing is tightly coupled with transcription and contains a series of steps including 5’-end capping, splicing, 3’-end cleavage and polyadenylation (121,122). Proteomic studies identified many RNA processing factors as PRMT substrates (7,8,10-12). However, in most cases, the consequence of the methylation remains to be elucidated (67).

Several PRMTs are known to affect RNA splicing. PRMT4 methylates the transcriptional elongation factor CA150 and splicing factors SAP49, SmB and U1C at the proline-, glycine- and methionine-rich (PGM) motif (123,124). The subsequent analysis showed that PRMT4 promotes exon skipping of an exogenous splicing reporter and the endogenous CD44 gene (124). Similarly, PRMT6 prevents exon inclusion of endogenous vascular endothelial growth factor (VEGF) and spleen tyrosine kinase (Syk) transcript in an enzymatic activity-dependent manner (125), suggesting PRMT6 could also methylate splicing factors.

The splicing function of PRMT5 and PRMT7 is linked to the methylation of Sm proteins (19,126-128). In the cytoplasm, the survival motor neuron (SMN) protein binds to the dimethylated Sm proteins to form the core protein structure for small ribonucleoprotein (snRNP) assembly (129). The mature snRNPs are imported into the nucleus and incorporated into spliceosome, the macromolecular machinery that carries out pre-mRNA splicing. Inhibition of SMN-Sm protein interaction leads to defective snRNP assembly and pre-mRNA splicing (129,130).

PRMT9 is a newly characterized type II PRMT that specifically methylates splicing factors SAP145/SF3B2 and SAP49 (20). Transcriptome analysis identified changes in alternative splicing in the PRMT9 knockdown cells, indicating that PRMT9 is a non-histone arginine methyltransferase that regulates RNA splicing.

Heterogeneous nuclear ribonucleoproteins (hnRNPs) and the serine/arginine-rich (SR) proteins are also the targets of PRMTs (67). HnRNPs are a class of RNA binding proteins that are involved in pre-RNA splicing and mRNP packaging for nuclear export. The functional consequences of arginine methylation of hnRNPs are mostly unknown. However, in several cases, inhibition of arginine methylation changes intracellular localization and RNA binding ability of hnRNPs (131-135).

The SR proteins are conserved, non-snRNP splicing proteins that are involved in multiple steps of RNA splicing. The SR proteins contain one or two RNA-recognition domains (RRM) and a C-terminal RS-rich domain. The RRM domain binds to RNA, whereas the RS-rich domain interacts with both protein and RNA. SF2/ASF protein is a splicing factor that has additional roles in RNA export, surveillance and translation. Substitution of arginine with alanine at three methylation sites increased cytoplasmic localization of SF2/ASF and caused defects in splicing and nonsense-mediated decay, a RNA surveillance pathway (136). Cells treated with adenosine dialdehyde (AdOx), a AdoHcy hydrolase inhibitor, led to the accumulation of SFRS9/SRp30c in nucleoli (137). Therefore, arginine methylation of SR proteins determines their intercellular localization and function in RNA processing.

1.4.4 Translation

Arginine methylation occurs on proteins associated with the translation machinery (7,8,11,12). However, the consequences of the methylation are mostly uncharacterized. There are several reports that provide links between PRMTs and translation. PRMT3 and PRMT5 methylate different ribosomal subunits (42,138). Disruption of the PRMT3 gene in yeast resulted in the imbalance of the 40S:60S ribosomal subunit ratio (139). However, the reduction of PRMT3 levels in mouse cells had no effect (42). PRMT5 methylates ribosomal protein S10 (RPS10). Mutation at the methylation sites decreased its association with ribosomes and caused defective protein synthesis (138). Therefore, PRMTs can affect translation via the association with and methylation of the translation machinery.

1.5 Reversal of arginine methylation

1.5.1 Dynamic nature of arginine methylation

The dynamic nature of arginine methylation has been demonstrated (65). For instance, estrogen stimulation results in a transient increase of ERα methylation in MCF7 cells (100). The level of methylation is at its peak within 5 minutes after estrogen treatment and then declines rapidly. Another study showed that estrogen stimulation leads to histone H3R17 methylation at the estrogen-responsive pS2 promoter in a cyclic manner (140). Other study showed that the symmetric dimethylation on histone H4R3 at gene promoters was erased as the genes were derepressed (31). A rapid change in monomethylation on cellular proteins upon

transcription inhibition was uncovered in a proteomic study (9). These findings suggest that active arginine demethylation occur in nature, and that could be a consequence of the rapid turnover of the modified proteins or enzymatic reaction by uncharacterized demethylases.

1.5.2 Putative arginine demethylase JMJD6

JMJD6 is the only enzyme that has been shown to demethylate methylarginine on both histones (141,142) and non-histone proteins (143-148). It belongs to the family of iron- and 2-oxoglutarate-dependent oxygenases. The family members share a conserved catalytic domain composed of a double-stranded β-helical fold. The catalytic domain was named JmjC domain because of the founding member *jmj* gene. The JmjC domain proteins target a broad range of substrates and participate in epigenetic regulation during development and in diseases (149).

JMJD6 was reported to erase the methyl marks on histone H3R2 and H4R3 (141). This was supported by western blot and MS-based analysis on bulk histones and histone peptides incubated with or without the enzyme. Additionally overexpression of the V5-tagged wild-type JMJD6 but not the iron-binding mutant reduced the overall methylation of H3R2 and H4R3 in HeLa cells. Other studies also suggested a demethylase function of JMJD6 for histone H4R3 (142,150). However, these findings were challenged by the studies showing that JMJD6 instead catalyzes lysine hydroxylation on histones and splicing factors (151,152).

The conflicting history of JMJD6's catalytic activity is also extensively reviewed (153).

The importance of JMJD6 in development was suggested by the knockout mice studies. Three JMJD6 deficient mouse lines have been independently reported (154-156). JMJD6 deficient fetus showed severe differentiation defects in multiple tissues including eyes, heart, lung, kidney, intestine, and defective erythropoiesis and T lymphopoiesis, eventually leading to perinatal lethality. However, the mechanisms by which JMJD6 regulate tissue development are still not known. Moreover, the tissue specific roles of JMJD6 in both embryos and adult animals are not identified, largely due to the lack of tissue specific knockout or knockdown models.

Recent evidences suggest that JMDJ6 is a versatile regulator for gene expression. JMJD6 modifies histones via arginine demethylation and lysine hydroxylation (141,142,152). It is tempting to hypothesize that JMJD6 antagonizes the action of PRMTs, especially PRMT1, PRMT5, PRMT6 and PRMT7, because these PRMTs methylate histone H3R2 and H4R3. Another study showed that lysine hydroxylation by JMJD6 has an inhibitory effect on acetylation and methylation on the same residues, and *vice versa* (152), suggesting a crosstalk between these modifications on histones. At the level of transcriptional regulation, JMJD6 controls RNA polymerase II elongation via its association with the active P-

TEFb complexes and the dual demethylase activities of JMJD6 toward histone H4R3 and 7SK RNA at distal enhancers (142).

JMJD6 also catalytically modifies transcription factors including p53 (157), estrogen receptor α (ERα) (144), PAX3 (148), and heat-shock protein of 70 kDa (HSP70) (145) to modulate their transcriptional activity or chromatin binding. Hydroxylation of p53 by JMJDJ6 represses p53 transcriptional activity by promoting the association of p53 with its negative regulator MDMZ (157). In response to estrogen treatment, JMJD6 demethylates ERα in MCF7 human breast cancer cells (144). JMJD6 also removes the methylation on PAX3, a transcription factor that controls transcription and differentiation in muscle and melanocyte lineages (148). The arginine methylation of PAX3 appears to be crucial for the loading of PAX3 on mitotic chromosomes. HSP70 is methylated by PRMT4 and demethylated by JMJD6, which regulates retinoid acid-induced gene expression (145).

JMJD6 has an active role in RNA processing. It associates with multiple splicing regulatory proteins including U2 auxiliary factors (U2AFs) and SR proteins (151,158,159). Knockdown or over-expression of JMJD6 results in changes in alternative splicing (158,159). Notably, JMJD6 can regulate splicing of a constitutive splice reporter in a manner independent of catalytic activity (159), suggesting a scaffold function of JMJD6 in splicing. In addition to the association with RNA processing factors, JMJD6 is able to bind RNA in *vitro* (160) and in

cultured cells (159). Thus, JMJD6 might regulate RNA processing through direct binding with RNA and splicing proteins.

1.5.3 Citrullination counteracts arginine methylation

Protein citrullination is a posttranslational modification that converts peptidyl arginine to peptidyl citrulline. The reaction is also referred as deimination because it involves an exchange of an imine for a carbonyl group (1). The enzymes that catalyzes citrullination are named peptidylarginine deiminases (PADs) (161). The enzyme PAD4 was shown to convert monomethylarignine into citrulline, which removed the methyl group on guanidinium group (162). However, the subsequent studies determined that citrullination of methylarginine by PAD4 is inefficient (163). Moreover, other studies showed that methylation of the guanidinium group can even prevent it from citrullination (164,165). Therefore, citrullination likely serves as a mechanism that prevents arginine methylation rather than demethylation (166).

1.6 Adipocyte differentiation

1.6.1 Adipose tissues and functions

Adipose tissue is composed of various types of cells including adipocytes, fibroblasts, endothelial cells, macrophages, and mesenchymal stem cells (167). White adipose tissue (WAT) and brown adipose tissue (BAT) are the two main types of adipose tissue, which are distinct in their morphology and function

(167,168). A typical WAT has light-colored appearance and contains mature adipocytes with large unilocular lipid droplets. It serves as triglyceride storage site and an endocrine organ for energy homeostasis. In contrast, BAT has more capillaries than WAT. Brown adipocytes contain multilocular lipid droplets and a high content of mitochondria as compared to white adipocytes. The major role of BAT is to maintain body temperature and energy expenditure through thermogenesis.

In addition to morphology and function, WAT and BAT have differences in body distribution and developmental pathways (167,168). In human, WAT is located mainly in abdominal and subcutaneous depots (167,169,170). Other sites such as retro-orbital, periarticular, intramuscular, pericardial depots and bone marrow, also contain WAT. BAT is found in the interscapular regions of infants, and cervical, supraclavicular and paravertebral sites in adults (167). The size and distribution of both WAT and BAT can change with age and diseases, and depot-specific metabolic functions have also been studied (167).

WAT and BAT originate from mesoderm, however, a divergence between white and brown adipocyte precursor along the developmental pathway was shown by the lineage-tracing studies in mice (171). BAT shares a common $MYF5^{+}PAX7^{+}$ precursor with muscle cells, whereas WAT precursor cells were found in neighboring blood vessels within the mature fat depots. Furthermore, the timing of WAT and BAT development is different. WAT begins to develop in late

gestation and undergoes a rapid expansion in postnatal stage in response to nutrient uptake. By contrast, BAT generally expands in utero (168).

1.6.2 Adipocyte differentiation and cell culture models

Adipocyte differentiation, also referred to as adipogenesis, is a developmental process during which multipotent stem cells convert into mature adipocyte. It takes place not only in developing embryos but also in adults. A human study showed a 10% annual turnover rate in adult human adipose tissues (172). Therefore, adipocyte differentiation is crucial for maintenance of adipose tissues throughout the body. In obese adults, adipose tissues undergo a characteristic expansion resulting from the enlargement of adipocyte size (hypertrophy) and the increase in adipocyte number (hyperplasia) (173). Adipose expansion has been examined in an Adipochaser mouse model by a high-fat diet challenge (174). The study determined that hypertrophy occurs in epididymal and subcutaneous adipose tissues at the early stages of high-fat diet challenge. A prolonged exposure to the high-fat diet causes expansion in gonadal adipose tissues but not in the subcutaneous fat depots. These results not only confirmed that adipogenesis occurs in response to nutrition overload, but also highlight the differences in adipogenic potential of various adipose depots.

Adipocyte differentiation has been extensively studied using multiple cell models (175). Embryonic stem (ES) cells, mouse embryonic fibroblasts (MEFs), bone marrow and fat stromal derived cells are the major sources for the primary

cells. Moreover, MEFs or stromal cells can be immortalized by either 3T3 protocol or introduction of SV40 large T-antigen to allow sustained manipulation. Immortalized cell lines such as 3T3-L1, 3T3-F422A, C3H10T1/2 and OP9 differentiate spontaneously upon hormonal treatment (176-179). In contrast, the non-adipogenic cell lines such as NIH3T3 and other immortalized MEFs can be differentiated into adipocytes by introduction of adipogenic transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/Enhancer binding protein α and β (C/EBPα and C/EBPβ) (180-182).

1.6.3 Transcriptional regulation of adipocyte differentiation

The transcriptional network for adipocyte differentiation is centered on the nuclear receptor PPARγ (175,183), which is a transcription factor both necessary and sufficient for adipogenesis (181,184,185). The transcription of *Pparγ* gene gives rise to two protein isoforms, PPARγ1 and PPARγ2, through alternative splicing and promoter usage (186). Both isoforms promote adipogenesis in the PPARγ-null MEFs, despite the fact that PPARγ2 is more efficient (187). PPARγ is also required for maintenance of cellular phenotype and survival of mature adipocytes (188,189). Although many other adipogenic factors had been identified, no factor can function efficiently in the absence of PPARγ. Therefore, PPARγ is considered the master regulator of adipogenesis.

C/EBP family transcription factors have crucial roles in the adipogenic differentiation by either facilitating transcriptional activation of PPARγ or collaborating with PPARγ to activate downstream targets. C/EBPβ and C/EBPδ are induced early during differentiation by cAMP agonist 3-isoburyl-1-methlxanthine (IBMX) and glucocorticoid agonist dexamethasome (190). These two early pro-adipogenic transcription factors activate the expression PPARγ and C/EBPα through a direct binding at the promoters and enhancers (191,192). A double knockout mouse line showed a significant reduction in adipose tissues, whereas the lack of either C/EBPβ or C/EBPδ only had a partial reduction in adipose formation (193), supporting the idea that these two transcription factors cooperate to initiate adipocyte differentiation both *in vitro* and *in vivo*. Different from C/EBPβ and C/EBPδ, C/EBPα expression is relatively late during adipogenesis (190). C/EBPα collaborates with PPARγ to promote adipocyte differentiation and phenotype maintenance (194,195). It is also required for the insulin sensitivity in adipocytes (194).

Many other pro-adipogenic and anti-adipogenic transcriptional factors and cofactors have been characterized over the past two decades. These studies have also been extensively reviewed (168,175,183,196-198). Krupprl-like factors (KLF4, KLF5, KLF6 and KLF15), E2F family transcription factors (E2F1-3), early growth response-2 (EGR2, also known as Krox20), cAMP response element binding protein (CREB), glucocorticoid receptor (GR), signal transducer and activator of transcription 5A/B (STAT5A/B), retinoid X receptor (RXR) and sterol regulatory

element-binding protein 1c (SREBP-1c) are pro-adipogenic factors that stimulate PPARγ and C/EBPα expression. A series of negative regulators including E2F4, GATA2, GATA3, KLF2, KLF3, FOXO1 and β-catenin/TCF4 suppress PPARγ or C/EBPα expression. The co-regulators such as SWI/SNF chromatin remodelers, the Mediator complex, CBP/p300 acetyltransferases and MLL3/MLL4 methyltransferases facilitate chromatin remodeling and enhancer establishment for the expression of PPARγ and other adipogenic genes. In contrast, H3K9 methyltransferase G9a directly represses PPARγ expression. The co-repressor complexes NCoR and SMRT, and the Sirt1 NAD-dependent protein deacetylase are involved in the repression of PPARγ target genes.

Global transcription and epigenomic alterations during adipocyte differentiation were determined by a number of studies using genome-wide profiling approaches for RNA levels, histone modifications, and chromatin binding of transcription factors and cofactors in the differentiating adipocytes, particularly in 3T3-L1 adipocytes (192,195,199-205). The genome-wide distribution of various histone modifications such as H3K4me1/2/3, H3K27Ac, H3K27me3, H3K36me3 and H3K9Ac has been mapped in differentiating 3T3-L1 cells (199,200,202). These studies help to define the activity of gene promoters and enhancers. Changes in chromatin structure were determined by DNase site analysis in combination with high throughput DNA sequencing (DHS) (192). The results correlate with changes in gene expression and the cooperative binding of multiple transcription factors and cofactors at the specific genomic loci

(192,195,199,200,202-205). These findings greatly advance our knowledge on the mechanisms by which the adipogenic signals integrate at specific chromatin sites for initiation of the transcription program during differentiation.

1.6.4 PRMT in regulation of adipocyte differentiation

Among the nine members of PRMT family, PRMT4 and PRMT5 affect adipocyte differentiation (55,79). The PRMT4 knockout embryos developed less BAT at E18.5 as compared to the wild-type embryos (55). The PRMT4 knockout MEFs failed to differentiate into adipocytes. Knockdown of PRMT4 in 3T3-L1 preadipocytes also impaired their differentiation. The molecular analyses showed that PRMT4 co-activated PPARγ and was recruited to an endogenous adipocyte specific gene ap2/FABP4 (55), indicating that PRMT4 acts as a co-activator to promote PPARγ target gene expression. However, it is still unclear whether PRMT4 affects the expression of PPARγ and C/EBPs genes in the early stage of differentiation and *in vivo*.

PRMT5 is directly involved in both the activation of PPARγ and PPARγ target genes (79). Knocking down PRMT5 decreased the adipogenic potential of 3T3-L1 and C3H10T1/2 cells. In contrast, ectopic expression of PRMT5 promotes differentiation in these two cell lines. Introduction of either PPARγ or C/EBPα into the non-adipogenic NIH3T3 fibroblasts promoted adipogenic differentiation upon hormonal induction. However, when PRMT5 was depleted in the NIH3T3

fibroblasts, the cells failed to differentiate. More interestingly, the loss of PRMT5 prevented the binding of BRG1 at the PPARγ or C/EBPα target promoters and enhancers, suggesting that PRMT5 might assist the loading of BRG1-based SWI/SNF complexes at promoters and enhancers to facilitate gene transcription (79).

Due to the lack of mouse models, the *in vivo* adipocyte function of PRMT5 is unknown. Interestingly, deletion of *Copr5*, a binding partner of PRMT5, reduced the number of adipocytes in the retroperitoneal WAT in 8 and 16 week-old mice (206). The adipogenic differentiation of embryoid bodies and primary MEFs from the knockout mice was significantly delayed. Subsequent studies indicated that COPR5 and PRMT5 repressed the expression of *Dlk-1,* a negative regulator of adipogenesis (206). Thus, PRMT5 functions as both a co-activator and a co-repressor in a gene-specific manner during adipogenesis.

1.7 Outstanding questions and research scope

Arginine methylation is a post-translational modification crucial for many cellular and developmental processes. Although the functions and targets of PRMTs had been extensively studied, the connection between the molecular function of each PRMT and the biological events is not fully established. Moreover, it was proposed that JMJD6 is an arginine demethylase that antagonizes certain PRMTs. However, the enzymatic activity of JMJD6 is still contradictory. The

counteracting actions between PRMTs and JMJD6 should be carefully examined in a given biological process.

In this thesis, I describe my investigation of PRMT7 (Chapter II) and JMJD6 (Chapter III) in models for adipocyte differentiation. PRMT7 and JMJD6 are two distinct enzymes that potentially antagonize each other in arginine methylation and gene expression. By using the well-characterized differentiation models, I wished to determine whether and how these enzymes regulate gene expression and cellular differentiation. My work showed that PRMT7 is dispensable, whereas JMJD6 is necessary for adipocyte differentiation. The adipogenic function of JMJD6 is independent of its enzymatic activity. The characterization of the underlying mechanisms by which JMJD6 regulates gene expression during differentiation is also presented.

CHAPTER II

Characterization of PRMT7 in Adipocyte Differentiation

2.1 Abstract

Protein arginine methyltransferase 7 (PRMT7) is categorized as a type II and type III enzyme that produces symmetric dimethylated arginine and monomethylated arginine, respectively. However, the biological role of PRMT7 is not well characterized. We previously showed that PRMT5, a type II PRMT that associates with BRG1-based SWI/SNF chromatin remodeling complex, is required for adipocyte differentiation. Since PRMT7 also associates with BRG1-based SWI/SNF complex and modifies core histones, we hypothesized that PRMT7 might play a role in transcriptional regulation of adipogenesis. In the present study, we determined that the expression of PRMT7 did not change throughout adipogenic differentiation of C3H10T1/2 mesenchymal cells. Knockdown or over-expression of PRMT7 had no effect on lipid accumulation or adipogenic gene expression in differentiating C3H10T1/2 cells or in C/EBPα-reprogrammed NIH3T3 fibroblasts. Based on these results, we conclude that PRMT7, unlike PRMT5, is dispensable for adipogenic differentiation in tissue culture models.

2.2 Materials & Methods

2.2.1 Cell line

Mouse C3H10T1/2 and NIH3T3 cells were obtained from the ATCC. C3H10T1/2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Sigma) and 100U/ml of penicillin/streptomycin (Invitrogen). NIH3T3 cells were maintained in DMEM high glucose with 10% calf serum (Sigma). 293T and BOSC23 cells were obtained from Dr. S.N. Jones (UMass Medical School) and Dr. R.E.Kingston (Massachusetts General Hospital), respectively, and were grown in the same medium as C3H10T1/2 cells. For adipogenic differentiation, two-day postconfluent cells were differentiated with DMEM medium containing 10% FCS, 10µg/ml insulin, 0.5mM 3-isobutyl-1-methyxanthine, 1µM dexamethasone, and 10µM troglitazone (Sigma). After 48 hours incubation, media on the differentiating cells was replaced with media containing 5µg/ml insulin. Subsequently, the media was changed every other day until harvest. To evaluate cell proliferation, $1x10^5$ cells were seeded in 6-well plates (Corning Inc.), and the number of viable cells was counted under a microscope (CK2, Olympus) each day from day 1 to day 4 with a hemocytometer (Hausser Scientific).

2.2.2 Plasmid DNA construction

pENTR/pTER+ vector and pLentiX2 Dest vector were gifts from Dr. Eric Campeau (UMass Medical School). The preparation of lentiviral small hairpin RNA

(shRNA) constructs was done as previously described (31,188). Briefly, shPRMT7-1, shPRMT7-2 and scrambled control oligonucleotides were cloned into a pENTR/pTER+ vector. These constructs were individually incubated with the pLentiX2 DEST vector and LR clonase II enzyme mix (Invitrogen) to generate pLentiX2 DEST/ shPRMT7-1, pLentiX2 DEST/shPRMT7-2 and pLentiX2 DEST/shCtrl constructs. These lentiviral constructs were amplified in Stbl3 competent cells (Invitrogen) for generating lentiviruses. The pBABE puromycin empty vector (pBABE vector), FLAG-tagged PRMT7 construct (pBABE PRMT7) and pBABE CEBPα construct were previously described (31,189) and were individually amplified in TOP10 competent cells (Invitrogen) for generating retroviral DNA as previously described (31,189).

2.2.3 Virus preparation and transduction

The preparation of viruses was performed as previously described (188,190). Briefly, for lentiviruses, the packaging vectors pLP1, pLP2, pVSVG (Invitrogen) and pLentiX2 DEST/shRNA constructs were co-transfected into 293T cells with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. BOSC23 cells were used for pBABE-based retrovirus production. After 48 hours incubation, the supernatant was harvested and filtered through 0.45µm syringe filter (Millipore). For viral infection, 1ml of the filtered supernatant and 4µg/ml of polybrene (Sigma) were used to infect one million cells. After 48 hours incubation, virus infected cells were selected in 2.5µg/ml puromycin (Invitrogen).

2.2.4 Protein expression analysis

Cells were washed twice with cold PBS and were harvested in RIPA buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA, 1% Nonidet P-40 and 0.25% sodium deoxycholate) supplemented with protease inhibitor cocktail (Roche). The samples were sonicated at high intensity setting for 3 minutes with 30sec on/off cycle in a Bioruptor (UCD-200, Diagenode). After quantifying the protein concentration by means of a Bio-Rad protein assay, the protein samples were then mixed with 4X SDS loading buffer (240mM Tris-HCl pH6.8, 8% SDS, 40% glycerol, 0.01% bromophenol blue and 10% β-mercaptoethanol) and boiled at 95°C for 10min. 30µg protein samples were separated on 10% SDSPAGE and transferred onto PVDF membrane (Bio-Rad). The blots were blocked overnight in 3% non-fat milk (Essential Everyday). The next day, proteins were detected using specific antibodies (1:1000 dilution) and HRP-conjugated secondary antibodies (1:2000 dilution). The rabbit polyclonal antibodies against human PRMT7 (sc-98882) and rat C/EBPα (sc-61) were purchased from Santa Cruz Biotechnology. The mouse monoclonal antibody against mouse PPARγ (sc-7273) and the goat polyclonal antibody against human Prmt5 (sc-22132) were also purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-PI3K (ABS233) antibody was from Millipore. The secondary antibodies (NA9340 and NA9310) were purchased from GE Healthcare Life Sciences. The blots were developed on X-ray films with ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences). The

signal intensity was quantified by ImageJ.

2.2.5 Gene expression analysis

Total RNA was isolated from samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was prepared from 1µg of total RNA by Superscript III reverse transcriptase kit (Invitrogen). Quantitative PCR was performed on StepOne Plus real-time PCR machine with Fast SYBR Green Master mix (Applied Biosystems). The specific primers for gene expression analysis were listed in **Table A1**. Relative expression levels were determined by the comparative Ct method (207).

2.2.6 Oil Red O staining

Differentiating cells were washed once with PBS and fixed in 10% phosphate-buffered formalin (Fisher Scientific) overnight. The next day, the fixed cells were washed with 60% isopropanol and air dried completely. The cells were then stained with 60% Oil Red O (AMRESCO) for 10 minutes and washed repeatedly with tap water to remove excess stain. To quantify staining, Oil Red O was extracted from the cells with 100% isopropanol, and the optical density was measured at 500nm (OD500).

2.3 Results

2.3.1 Expression of PRMT7 remains constant during differentiation

The C3H10T1/2 cell line was established from C3H mouse embryos and has served as a faithful cell culture model for mesenchymal lineage differentiation (208-210). C3H10T1/2 cells can be differentiated into mature adipocytes by treating the confluent cells with a cocktail that contains insulin, dexamethasone, 3-isobutyl-1-methyxanthine (IBMX) and PPARγ ligands such as Troglitazone (211). Using this model, we first examined PRMT7 protein levels during adipogenic differentiation by Western blot analysis. We found that PRMT7 protein levels are relatively constant from the onset of differentiation (day 0) through the day 6 post-differentiation (**Figure II-1A and Figure II-1B**). We concluded that PRMT7 protein levels were not altered in differentiating C3H10T1/2 cells.

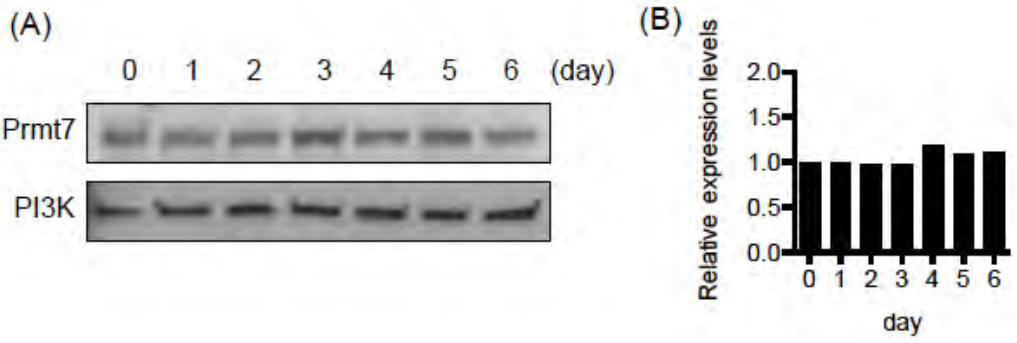

Figure II-1. The expression of PRMT7 remains constant in the differentiating C3H10T1/2 cells.
(A) Western blot analysis of protein extracts from day 0 to day6 post-differentiated C3H10T1/2 cells. Duplicate blots were probed with anti-PRMT7 antibody and with anti-PI3K antibody as a loading control. **(B)** The quantification of **(A)** by Image J. The levels of PRMT7 were normalized to PI3K loading control and presented as the relative expression levels to the

day 0 sample (day 0 = 1). The data represent the average of two independent experiments (n=2).

2.3.2 PRMT7 has no effect on cell proliferation

To study the function of PRMT7, we used viral vectors to knock down or over-express PRMT7 in C3H10T1/2 cells. Two lentiviral constructs (pLentiX2 DEST/shPRMT7-1 and pLentiX2 DEST/shPRMT7-2) that encode shRNAs against PRMT7 mRNA were used for knocking down endogenous PRMT7 in proliferating C3H10T1/2 cells. A pBABE retroviral construct (pBABE-PRMT7) encoding FLAG-tagged PRMT7 was used to over-express PRMT7. The virus-infected cells were selected with puromycin and the levels of PRMT7 in the selected cells were examined by Western blot analysis (**Figure II-2A**). Endogenous PRMT7 levels were reduced 10-fold or more in the knockdown cells compared to the scrambled shRNA control cells (**Figure II-2B**). PRMT7 levels were increased more than 5-fold in the FLAG-tagged PRMT7 over-expression cells compared to the pBABE empty vector control (**Figure II-2B**). Since PRMT5 is the major type II arginine methyltransferase and is also associated with SWI/SNF complexes (76), we examined PRMT5 protein levels in our samples. We observed no changes in PRMT5 levels in PRMT7 knockdown and overexpression C3H10T1/2 cells (**Figure II-2A**).

Both PRMT7 and PRMT5 exhibit type II arginine methyltransferase activity. However, unlike PRMT5, PRMT7 has no effect on cell proliferation in NIH3T3 fibroblasts (31). We measured the cell proliferation rate of PRMT7 knockdown and

over-expression C3H10T1/2 cells, and found that neither the reduction of PRMT7 nor the over-expression of PRMT7 affected the proliferation of C3H10T1/2 cells (**Figure II-3**). This result is consistent with the results from the previous study on NIH3T3 fibroblasts (31).

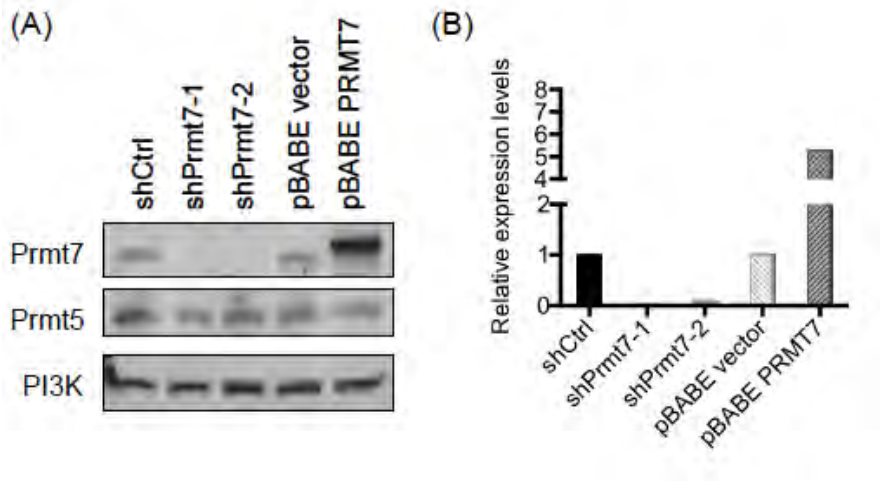

Figure II-2. Knockdown and over-expression of PRMT7 in C3H10T1/2 cells.
(A) A representative Western blot analysis from proliferating C3H10T1/2 cells with PRMT7 knockdown and over-expression. Endogenous PRMT7 was specifically depleted by two different lentiviral shRNA constructs (shPrmt7-1 and shPrmt7-2). The scrambled shRNA lentiviral construct (shCtrl) was used as a control. The pBABE retroviral construct encoding FLAG-tagged PRMT7 (pBABE-PRMT7) was used to ectopically express PRMT7, and the pBABE empty vector (pBABE vector) was used as a control. The blot was probed with anti-PRMT7 and anti-PRMT5 antibodies, and re-probed with anti-PI3K antibody as a loading control. **(B)** The quantification of PRMT7 in **(A)** by ImageJ. The levels of PRMT7 were normalized to the PI3K loading control and are presented as expression levels relative to the scrambled shRNA control or pBABE empty vector control. The data represent the average of two independent experiments (n=2).

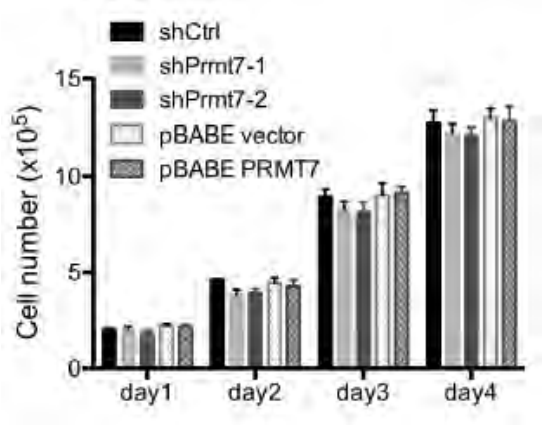

Figure II-3. PRMT7 has no effect on cell proliferation.
The growth rates of control (shCtrl and pBABE vector), PRMT7 knockdown (shPRMT7-1 and shPRMT7-2) and PRMT7 over-expression (pBABE PRMT7) C3H10T1/2 cells were measured by seeding 1×10^5 cells in 6-well plates, and the viable cells were counted each day for 4 days after seeding. The data represent the average of two independent experiments (n=2) counted in duplicate. Error bars show the standard deviation.

2.2.3 PRMT7 is not required for adipogenic differentiation

To determine whether PRMT7 affects adipogenesis, the PRMT7 knockdown and over-expression C3H10T1/2 cells were grown to confluence and treated with the differentiation cocktail. At day 6 post-differentiation, the accumulation of intracellular neutral lipids was measured by Oil Red O staining. The Oil Red O staining showed similar levels of lipid accumulation in PRMT7 knockdown as well as PRMT7 overexpression cells as compared to the control cells. (**Figure II-4A** and **Figure II-4B**). This result suggests that the changes in PRMT7 levels did not affect lipid accumulation in differentiating C3H10T1/2 cells.

PPARγ and C/EBPα are the key transcription factors for adipogenic differentiation and for the maintenance of the adipocyte phenotype

(180,181,195,205). We examined the protein levels of PPARγ and C/EBPα in day 6 post-differentiation cells by Western blot analysis and found no significant difference in either PRMT7 knockdown or PRMT7 over-expression cells compared to the corresponding controls (**Figure II-4C**). In addition, to rule out the possibility that PRMT7 functions as a cofactor of PPARγ and C/EBPα, we measured the mRNA expression levels of PPARγ and C/EBPα target genes in day 6 post-differentiation samples by real-time quantitative PCR (**Figure II-4D**). We found that PRMT7 has no significant impact on fatty acid synthase (*Fasn*), adiponectin (*AdipoQ*), and fatty acid binding protein 4 (*Fabp4*) gene expression in the differentiating cells. These results suggest that PRMT7 is dispensable for adipogenic gene expression.

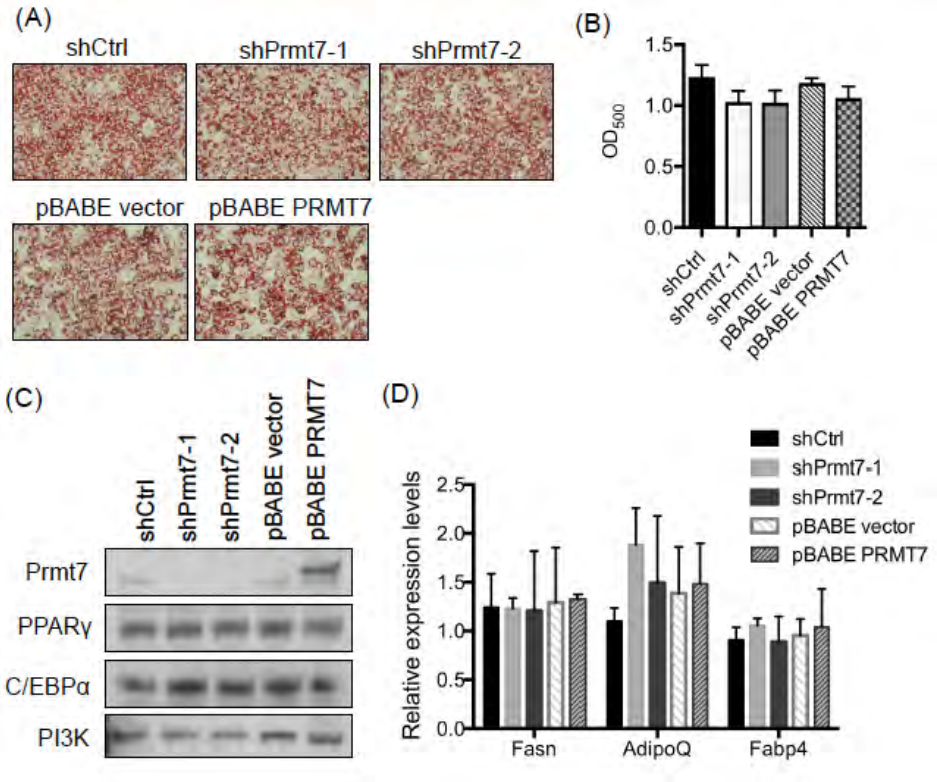

Figure II-4. PRMT7 has no effect on the adipogenic differentiation of C3H10T1/2 cells.

(A) Representative Oil-Red O staining images of day 6 post-differentiated C3H10T1/2 cells in which Prmt7 was either knocked down (shPrmt7-1 and shPrmt7-2) or over-expressed (pBABE PRMT7). The Prmt7 knockdown and over-expression C3H10T1/2 cells were grown to confluence and differentiated 48 h later. At day 6 post-differentiation, the cells were fixed with 10% formalin and stained with Oil-Red O. **(B)** The quantification of **(A)**. The Oil-Red O stain was extracted with 100% isopropanol and the optical density at 500nm (OD_{500}) was determined. The data represent the average of two independent experiments (n=2) assayed in duplicate. Error bars show standard deviation. **(C)** A representative Western blot analysis of endogenous Prmt7, PPARγ and C/EBPα expression in day 6 post-differentiated C3H10T1/2 cells. The levels of PI3K are presented as a loading control. **(D)** Gene expression analysis on day 6 post-differentiated C3H10T1/2 cells. The mRNA levels of fatty acid synthase (*Fasn*), adiponectin (*AdipoQ*) and fatty acid binding protein 4 (*Fabp4*) were measured by RT-qPCR. The individual mRNA levels were normalized to 5S rRNA. The normalized expression levels of the control cells were set as 1. The data are presented as the average of relative expression levels from two independent experiments (n=2) assayed in duplicate. Error bars show standard deviation.

Previous studies had shown that ectopic expression of PPARγ or C/EBPα alone in non-adipogenic NIH3T3 cell line reprograms NIH3T3 fibroblasts into adipocyte-like cells (180,181). To test if PRMT7 is required for the reprogramming of NIH3T3 fibroblasts, we first knocked down and over-expressed PRMT7 in NIH3T3 fibroblasts by using the same viral constructs that we used in C3H10T1/2 cells, and confirmed the knockdown and over-expression of PRMT7 by Western blot analysis (**Figure II-5A**). These cells were further infected with the retroviruses encoding C/EBPα at 70 % confluence. After the cells reached confluence, the differentiation cocktail was added to stimulate adipogenic differentiation. At day 6 post-differentiation, the accumulated lipid was evaluated by Oil Red O staining (**Figure II-5B**). We found that neither knockdown nor over-expression of PRMT7 caused a significant difference in Oil Red O staining in C/EBPα-reprogrammed NIH3T3 fibroblasts, which is consistent with the results in C3H10T1/2 cells.

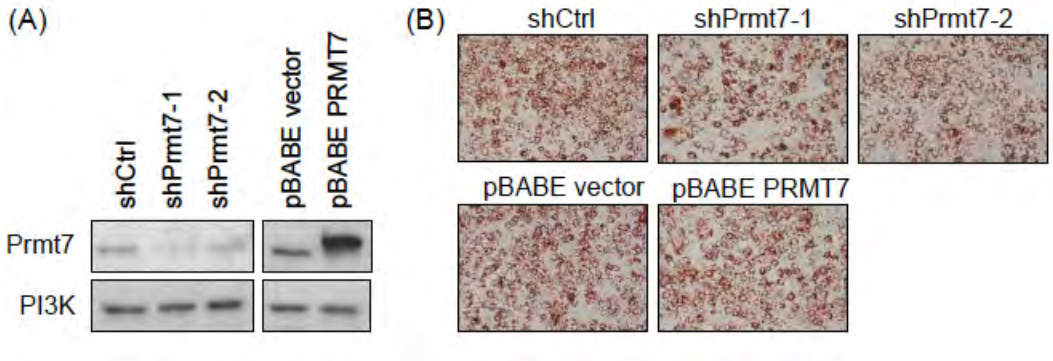

Figure II-5. PRMT7 does not affect C/EBPα-mediated reprogramming of NIH3T3 fibroblasts.
(A) Western blot analysis on PRMT7 knockdown and over-expression NIH3T3 fibroblasts. Endogenous PRMT7 was specifically depleted by the

lentiviral shRNA constructs (shPRMT7-1 and shPRMT7-2). The scrambled shRNA lentiviral construct (shCtrl) was used as a control. The pBABE retroviral construct encoding FLAG-tagged PRMT7 (pBABE PRMT7) was used to ectopically express PRMT7 and the pBABE empty vector was used as a control. The blot was probed with anti-Prmt7 antibody, and the PI3K levels are presented as a loading control. **(B)** Oil-Red O staining images of C/EBPα-reprogrammed NIH3T3 fibroblasts at day6 post-differentiation. Prmt7 knockdown and over-expression NIH3T3 fibroblasts were infected with retroviruses encoding C/EBPα at 70% confluence. Two day post-confluent cells were differentiated. At day 6 post-differentiation, the cells were fixed with 10% formalin and stained with Oil-Red O.

2.4 Discussion

Changes in gene expression during cell differentiation require alterations in higher-order chromatin organization as well as in local chromatin structure. Cells possess histone modifying enzymes and ATP-dependent chromatin remodeling enzymes to facilitate chromatin changes. The interplay between these two families of enzymes has been shown to be crucial for both transcription activation and repression (76,92,212). PRMT7 was identified as a histone arginine methylating enzyme (23,25,26) and was shown to associate with BRG1-based SWI/SNF ATP-dependent chromatin remodeling complex (31). These findings led us to investigate the possible roles of PRMT7 in adipogenic differentiation, which is a process that requires the function of BRG1-based SWI/SNF complex (213). Our data clearly showed that PRMT7 levels were significantly changed in the knockdown or overexpression cells, but manipulation of PRMT7 levels did not cause a differentiation deficiency. It is established that BRG1-based SWI/SNF complex is recruited to the adipogenic promoters upon differentiation. However, whether PRMT7 associates with BRG1-based SWI/SNF complex at adipogenic promoters is still unknown. Since PRMT7 has no effect on adipogenic gene expression, we expect that PRMT7 is not recruited to adipogenic promoters. Alternatively, even if there is binding, the function of PRMT7 is dispensable at these loci.

Functional redundancy within the PRMT family has not been characterized. PRMT7 was classified as a type II and a type III arginine methyltransferase by

characterization of its *in vitro* catalytic activity (22,23,25,26). Whether other PRMTs functionally compensate for PRMT7 is still unknown. The predominant type II arginine methyltransferase PRMT5 catalyzes the formation of MMA and SDMA in a nonprocessive fashion (18,214). Type I arginine methyltransferases also produce MMA (13,16,215). It is possible that PRMT5 or type I PRMTs partially or fully compensate for the loss of PRMT7 in the cells. Our data showed that PRMT7 knockdown or over-expression has no effect on PRMT5 protein levels in C3H10T1/2 cells. This observation is consistent with the results from the previous study in HeLa cells (128). However, we still cannot rule out the possibility that PRMT5 compensates for PRMT7 enzyme activity, even though PRMT5 protein levels remain constant. Further investigation is needed to address the possible crosstalk between PRMT5 and PRMT7 in chromatin regulation.

Whether changes in PRMT7 levels cause any developmental deficiencies *in vivo* remains to be elucidated. Several studies using cell lines or tissues have revealed regulatory roles for PRMT7 in tissue-specific gene expression. For example, PRMT7 negatively regulates neuronal differentiation of a human embryonal carcinoma cell line by repressing the expression of differentiation-specific genes (87). In mouse germ cells, PRMT7 was recruited to the imprinting control region through physical interaction with CTCFL, a testis-specific nuclear protein, and repressed imprinted gene expression (27). Furthermore, mouse embryonic stem cells and germ cells have relative high levels of PRMT7 compared with mouse embryonic fibroblasts (216,217). This evidence suggest that PRMT7

might have important functions in the maintenance of stem cell pluripotency and that the down-regulation of PRMT7 might be required for early cell fate determination. Taken together, these data suggest that PRMT7 might have a role during early development.

CHAPTER III

Roles of JMJD6 in Adipocyte Differentiation

3.1 Abstract

JMJD6 is a nuclear protein involved in histone modification, transcription, and RNA processing. Although JMJD6 is crucial for tissue development, the link between its molecular functions and its roles in any given differentiation process is unknown. We report that JMJD6 is required for adipogenic gene expression and differentiation in a manner independent of Jumonji C domain catalytic activity. JMJD6 knockdown led to a reduction of C/EBPβ and C/EBPδ protein expression without affecting mRNA levels in the early phase of differentiation. However, ectopic expression of C/EBPβ and C/EBPδ did not rescue differentiation. Further analysis demonstrated that JMJD6 was associated with the *Pparγ2* and *Cebpα* loci and putative enhancers. JMJD6 was previously found associated with bromodomain and extra-terminal domain (BET) proteins, which can be targeted by the bromodomain inhibitor JQ1. JQ1 treatment prevented chromatin binding of JMJD6, *Pparγ2* and *Cebpα* expression, and adipogenic differentiation, yet had no effect on C/EBPβ and C/EBPδ expression or chromatin binding. These results indicate dual roles for JMJD6 in promoting adipogenic gene expression program by post-transcriptional regulation of C/EBPβ and C/EBPδ and direct transcriptional activation of *Pparγ2* and *Cebpα* during adipocyte differentiation.

3.2 Materials and Methods

3.2.1 Cell culture

Mouse C3H10T1/2, 293T, and BOSC23 cells were maintained in growth media consisting of DMEM high glucose medium (Invitrogen), 10% fetal calf serum (FCS, Sigma) and 100 U/ml penicillin/streptomycin (Invitrogen). For adipogenic induction, confluent cells were cultured in DMEM high glucose medium supplemented with 10% FCS, 10 µg/ml insulin, 0.5 mM IBMX, 1 µM dexamethasome, and 5 µM Troglitazone (all reagents from Sigma) for 48 h. After induction, the differentiating cells were maintained in DMEM high glucose medium supplemented with 10% FCS and 5 µg/ml insulin. Media was changed every other day until harvest.

3.2.2 Plasmid DNA construction

pENTR/pTER+ vector and pLentiX2 DEST vector were gifts from Dr. Eric Campeau. The preparation of small hairpin RNA (shRNA) lentiviral constructs was performed as previously described (218). Briefly, JMJD6 shRNAs, GFP shRNA and scrambled sequence shRNA oligonucleotides were individually cloned into pENTR/pTER+ vector. The pENTR/pTER+ shRNA constructs were subsequently incubated with LR clonase II enzyme mix (Invitrogen) and pLentiX2 DEST vector containing a puromycin resistance gene to generate pLentiX2 DEST/sh*Jmjd6*, pLentiX2 DEST/shGFP, and pLentiX2 DEST/shCtrl constructs. The sequences of shRNA oligonucleotides are listed in **Table A1**. To generate the constructs for

JMJD6 and HIF1AN protein expression, the coding sequence of JMJD6 and HIF1AN with a C-terminal FLAG tag sequence was amplified from mouse embryonic fibroblast cDNA by RT-PCR and cloned into pBABE retroviral vector containing a blasticidin resistance gene (219) and pcDNA3.1(-) vector (Invitrogen). The construct for the catalytic-inactive mutant JMJD6 (H187A/D189A) protein (151) was generated from the wild type JMJD6 construct by QuikChange Lightning site-directed mutagenesis kit (Agilent). The primers for cloning and mutagenesis are listed in **Table A1**.

3.2.3 Virus preparation and transduction

Lentivirus preparation was performed as previously described (218). Briefly, the shRNA lentiviral constructs were co-transfected with pLP1, pLP2 and pVSVG packaging vectors into 293T cells with Lipofectamine 2000 reagent (Invitrogen). The constructs encoding wild type JMJD6 (JMJD6-wt) and the catalytic-inactive mutant JMJD6 (JMJD6-mut) were individually transfected into BOSC23 cells (220). The viral supernatant was harvested after 48 h incubation and filtered through 0.45 µm syringe filter (Millipore). To infect C3H10T1/2 cells, 2 ml of the filtered supernatant supplemented with 4 µg/ml of polybrene (Sigma) were used to infect one million cells for 48 h. The infected cells were then selected in media containing 2.5 µg/ml puromycin (Invitrogen) or 5 µg/ml of blasticidin (Invitrogen).

3.2.4 Oil Red O staining

Cells were washed once with PBS and fixed in 10% phosphate-buffered formalin (Fisher Scientific) overnight at room temperature. The next day, the cells were washed with 60% isopropanol and air dried completely. The cells were stained with 60% Oil-Red O (AMRESCO) for 10 min and washed extensively with running tap water. To quantify staining, Oil Red O was extracted with 100% isopropanol and measured at optical density 500 nm (OD_{500}).

3.2.5 Gene expression analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) and treated with DNaseI (Invitrogen) according to the manufacturer's instructions. cDNAs were prepared from 1 µg of total RNA with random hexamers (Roche) and the Superscript III reverse transcriptase kit (Invitrogen). Real-time quantitative PCR was performed on a StepOne Plus real-time PCR machine with Fast SYBR green master mix (Applied Biosystems). The relative gene expression levels were calculated as $2^{(Ct_{5S\ rRNA} - Ct_{gene})}$ and were normalized to the experimental control as indicated (221). The primers are listed in **Table A1**.

3.2.6 Protein expression analysis

Cells were washed twice with cold PBS and harvested in RIPA buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 0.25% sodium deoxycholate) supplemented with protease inhibitor cocktail (Roche). Mouse

adipose tissue extracts were prepared as described (222). Protein concentration was measured using Bio-Rad protein assay. The samples were mixed with 4X SDS loading buffer (240 mM Tris-HCl, pH6.8, 8% SDS, 40% glycerol, 0.01% bromophenol blue and 10% β-mercaptoethanol) and boiled at 95°C for 10 min. Samples were separated on 10% SDS-PAGE and transferred onto PVDF membrane (Bio-Rad). The blots were blocked in 3% non-fat milk overnight at 4°C. After a sequential incubation with the primary antibodies and HRP-conjugated secondary antibodies, the blots were developed on X-ray films with ECL Western blotting detection reagents (GE Healthcare Life Sciences). Primary antibodies against JMJD6 (sc-11366), JMJD6 (sc-28348), HIF1AN (sc-26219), PPARγ (sc-7273), C/EBPα (sc-61), C/EBPβ (sc-150), C/EBPδ (sc-151), and β-ACTIN (sc-81178) were purchased from Santa Cruz Biotechnology. PI3-kinase p85 (ABS233) antibody was purchased from Millipore. The phospho-C/EBPβ (#3084) antibody was purchased from Cell Signaling. The anti-FLAG rabbit antisera was described (171). The anti-BRD2 (A302-583A), anti-BRD3 (A302-367A), and anti-BRD4 (A301-985A50) antibodies were purchased from Bethyl Laboratories. The secondary antibodies (NA9340 and NA9310) were purchased from GE Healthcare Life Sciences. ImageJ software was used for image quantification.

3.2.7 Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as previous described (223). Briefly, cells were fixed in 1% formaldehyde at room temperature for 10 min

and harvested as cell pellets. The cell pellets were re-suspended in cell lysis buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10 mM EDTA, pH 8.0 and protease inhibitors) and sonicated in a Bioruptor (UCD-200, Diagenode) to obtain fragmented DNA sized around 500 bp. The samples were centrifuged at 12000 x *g* for 10 min at 4°C to remove insoluble debris. The supernatant containing the chromatin was pre-cleared with 40 µl of a 50% slurry protein A beads (Protein A Sepharose CL-4B, GE Healthcare Life Sciences) supplemented with 0.2 mg/ml salmon sperm DNA (Invitrogen) and 0.5 mg/ml BSA (Invitrogen). 50 µg of DNA were immunoprecipitated with 4 µg of specific antibody or 20 µl of serum overnight at 4°C. The next day, the immunocomplexes were incubated with 60 µl of a 50% slurry of protein A beads for 1 h at 4°C. After extensively washing, the immunocomplexes were eluted in 1% SDS and 1% $NaHCO_{3.}$ The eluted samples were subjected to reverse cross-linking for 4 h at 65°C. The immunoprecipitated DNA fragments were purified using the QIAquick PCR purification kit (QIAGEN) and used as templates for quantitative PCR. The fold difference of immunoprecipitated DNA fraction relative to input was calculated as $2^{(Ct_{input} - Ct_{ChIP})}$ (224). The primers are listed in Appendix 1. The antibodies for ChIP assays are C/EBPβ (sc-150), C/EBPδ (sc-151), RNA polymerase II (sc-899) and rabbit IgG control (sc-2027) from Santa Cruz Biotechnology. The anti-BRD4 (A301-985A50) antibody was from Bethyl Laboratories. The anti-JMJD6 rabbit sera was made against a bacterial expressed GST-fusion with human JMJD6 (aa 2-414), which was cloned by PCR-amplification and subsequently inserted into pGEX-2T

plasmid. The primers for cloning are listed in **Table A1**. The specificity of the JMJD6 antisera was evaluated by western blot as shown in **Figure A1**.

3.2.8 Immunoprecipitation

Cells were washed twice with cold PBS and lysed with a buffer containing 50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and protease inhibitor cocktail (Roche) on ice for 30 min. The cell lysate were pre-cleared by 40 µl of a 50% slurry of protein A beads. 1 mg of the cell lysate was incubated with either 20 µl of anti-JMJD6 rabbit antisera or rabbit IgG control (sc-2027) for 2 h at 4 °C. The samples were then incubated with 60 µl of a 50% slurry of protein A beads for 1 h at 4 °C. The immunocomplexes were eluted by adding 2X SDS loading buffer and subsequently boiled at 95 °C for 10 minutes before SDS-PAGE and subsequent Western blotting.

3.2.9 Small interfering RNA knockdown

ON-TARGETplus mouse *Cebpβ* and *Cepbδ* siRNA SMART pools and the non-targeting control siRNA pool were purchased from Dharmacon GE Healthcare Life Sciences. The target sequences for *Cepbβ* mRNA were GAGCGACGAGUACAAGAUG, CCUUUAGACCCAUGGAAGU, GCACCCUGCGGAACUUGUU, and GAAAAGAGGCGUAUGUAUA. The target sequences for *Cepbδ* mRNA were CGCGGAAGGAACACGGGAA, AGUUGUCGGCCGAGAACGA, GUAAGGAGAUGGACGCGUU, and

GGCACUGGACUGCGAGAGA. Briefly, cells were plated at a density of 1.5 x 10^5 cells per well in 12 well plates overnight and then transfected with siRNA pools using Lipofectamine 2000 in Opti-MEM medium (Invitrogen). After 4 h incubation, the medium was replaced with the normal growth medium for 20 h prior to adipogenic induction.

3.2.10 Plasmid and *In vitro* synthesized RNA transfection

pcDNA3-EGFP (#13031), pcDNA3.1(-) mouse C/EBPβ LAP (#12557) and pcDNA3.1(-) mouse C/EBPδ (#12559) were obtained from Addgene. For the preparation of *in vitro* synthesized RNA, pcDNA3.1(-) mouse C/EBPβ LAP, pcDNA3.1(-) mouse C/EBPδ and pcDNA3-EGFP plasmids were linearized with XbaI or HindIII and were *in vitro* transcribed with mMESSAGE mMACHINE T7 Ultra Transcription Kit (Ambion) according to the manufacturer's instructions. The capped and polyadenylated RNAs were further purified with RNA Clean and Concentrator Kit (Zymo Research). For transfection, 1.5 x 10^5 cells were plated in each well of 12-well plates overnight and transfected with either 2 μg of the plasmids or *in vitro* synthesized RNAs using Lipofectamine 2000 in Opti-MEM medium. After 4 h incubation, the media was replaced with the normal growth medium for 20 h prior to adipogenic induction.

3.2.11 Statistical Analysis

At least three independent experiments were performed as indicated in the Figure legends. The values are presented as the mean ± S.E.M. Statistical analyses were performed using Student's *t*-test with two-tailed distribution and equal variance.

3.3 Results

3.3.1 JMJD6 expresses in mouse adipose tissues and adipocytes

To understand the biological roles of JMJD6 in adipocyte differentiation, we first examined the expression of JMJD6 in adult adipose tissues and in differentiating adipocytes. Western blot analysis showed that JMJD6 protein is expressed in both white and brown adipose tissues from 6-week-old male and female C57BL/6 mice (**Figure III-1A**). For differentiating adipocytes, we used mouse multipotent mesenchymal C3H10T1/2 cells as a tissue culture model (225). C3H10T1/2 cells differentiate into adipocytes upon stimulation with an adipogenic induction media containing insulin, dexamethasone, 3-isobutyl-1-methylxanthine and Troglitazone. Induction of the master adipogenic regulators, PPARγ and C/EBPα, occurred on day 1 post-induction (**Figure III-1B**). JMJD6 is present in differentiating C3H10T1/2 adipocytes even before the onset of the differentiation (**Figure III-1B**). A transient increase of JMJD6 protein and mRNA expression was observed in the differentiating cells as compared to the levels at day 0 when the cells were undifferentiated (**Figure III-1C, D**). Previous work has shown that JMJD6 proteins form oligomers (226-229). Consistent with Western blot analysis and quantification of the monomer species (**Figure III-1B, C**), examination of a time course of differentiating cells indicated that the oligomeric form was present at the onset of differentiation and its level increased by days 2-3 post-differentiation and decreased thereafter (**Figure III-2**). Based on these results, we

concluded that JMJD6 is present in adult adipose tissues and expressed throughout the course of differentiation of C3H10T1/2 cells.

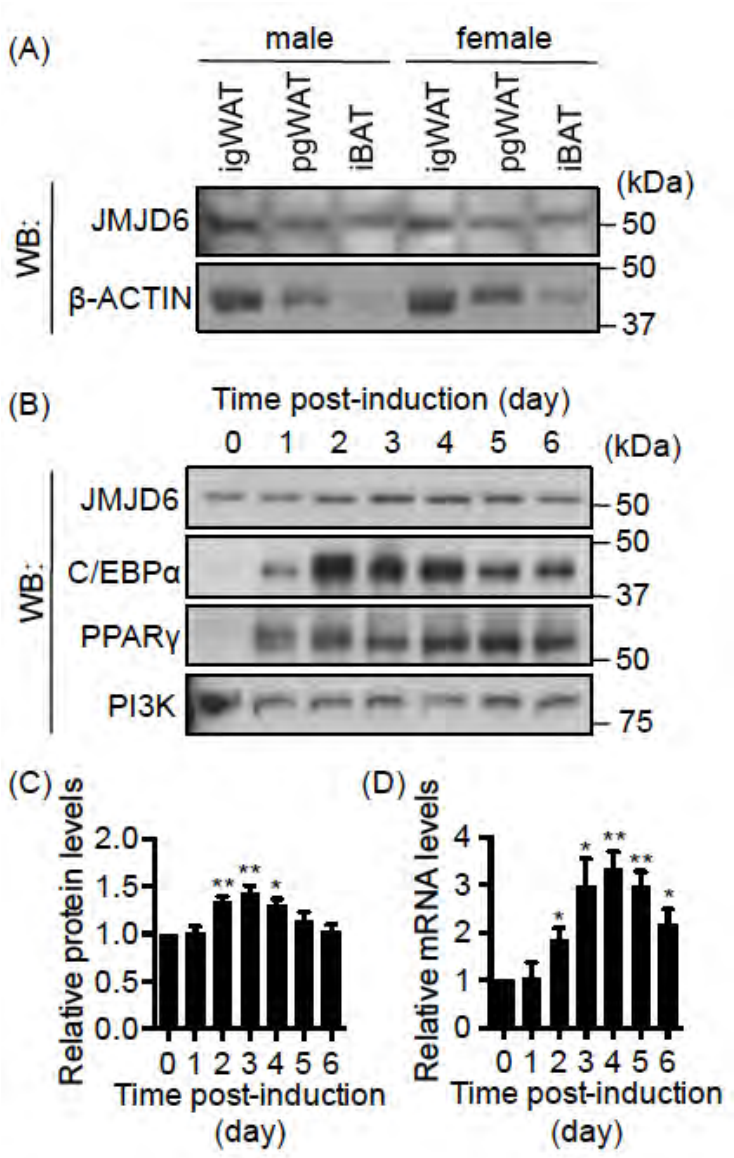

Figure III-1. JMJD6 is expressed in adipose tissues and differentiating C3H10T1/2 adipcoytes

(A) Representative western blots for JMJD6 and β-ACTIN protein levels in adipose tissues from 6-week-old male and female C57BL/6 mice. igWAT: inguinal white adipose tissue; pgWAT: perigonadal white adipose tissue; iBAT: interscapular brown adipose tissue. **(B)** Representative western blots for JMJD6, C/EBPα and PPARγ protein levels in undifferentiated (day 0) and in differentiating (day 1 to 6) C3H10T1/2 cells. PI3K levels were probed as a loading control. **(C)** Quantification of JMJD6 protein levels from three

independent western blots. The levels of JMJD6 were normalized to PI3K loading control and are presented as the relative expression levels to the day 0 sample (S.E.M, n=3, * p<0.05; ** p<0.01). **(D)** *Jmjd6* mRNA levels in the differentiating C3H10T1/2 cells were determined by real-time qPCR. The mRNA levels were normalized to 5s rRNA levels and the sample at day 0 was set as 1 (S.E.M, n=3, * p<0.05; ** p<0.01).

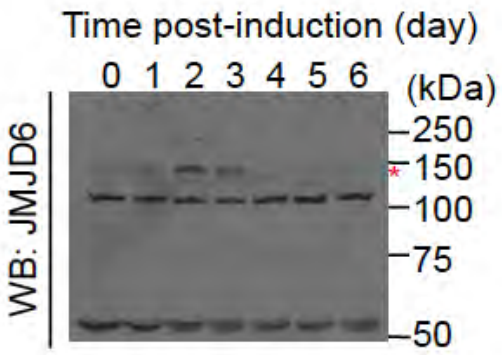

Figure III-2. JMJD6 oligomerization in the differentiating C3H10T1/2 cells.
A representative western blot for JMJD6 levels in undifferentiated (day 0) and in differentiating (day 1 to 6) C3H10T1/2 cells. The oligomeric JMJD6 protein bands (~130kDa) are indicated by the red asterisk.

3.3.2 Knockdown of JMJD6 impairs adipocyte differentiation

To investigate the function of JMJD6 in adipocyte differentiation, we knocked down JMJD6 in C3H10T1/2 cells by introduction of small hairpin RNAs prior to the onset of differentiation. Two shRNAs specific for JMJD6 were used. shJ6-2 targets the 5'UTR and the start of the 5' coding sequence, whereas shJ6-3 targets the 3'UTR of *Jmjd6* mRNA. The cells were induced to differentiate upon confluence. Adipogenic differentiation was evaluated at day 6 post-induction by quantifying the intracellular lipid content. Two distinct control shRNAs, a scramble sequence called shCtrl, and shGFP, had no effect on the adipogenic differentiation (**Figure III-3**). A significant reduction in lipid accumulation in the JMJD6

knockdown cells compared to the scramble shRNA control cells was observed (**Figure III-4A, B**), demonstrating that JMJD6 knockdown reduced the adipogenic potential of C3H10T1/2 cells. Moreover, the levels of the master regulators for terminal differentiation PPARγ and C/EBPα were significantly reduced in the day 6 post-induced JMJD6 knockdown cells (**Figure III-4C**). Consistent with the reduction in protein levels, *Pparγ* and *Cebpα* mRNA expression was significantly reduced in the JMJD6 knockdown cells (**Figure III-4D**). Other adipocyte-associated genes such as *Srebp1c*, *Fasn*, *AdipoQ* and *Fabp4* were also down-regulated (**Figure III-4D**). These results indicated that the knockdown of JMJD6 led to a failure of differentiation of C3H10T1/2 cells due to the inability of the cells to activate the adipogenic gene expression program.

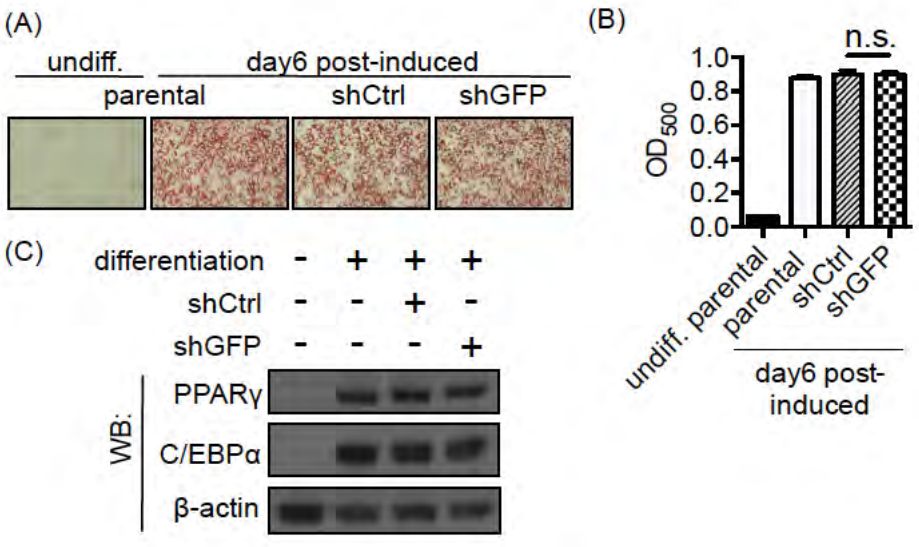

Figure III-3. Two distinct control shRNAs do not affect the adipogenic differentiaiton of C3H10T1/2 cells.
(A) Representative Oil Red O staining images, **(B)** the levels of Oil-Red O staining, and **(C)** the protein levels of PPARγ and C/EBPα from the cells as indicated.

3.3.3 JMJD6 regulates adipogenesis independently of its catalytic activity

To determine whether the catalytic activity of the JmjC domain in JMJD6 is required for adipocyte differentiation, we used a catalytically inactive mutant JMJD6 that includes substitution of histidine 187 and aspartate 189 with alanine (151). C3H10T1/2 cells were first transduced with the retroviral constructs encoding FLAG-tagged wild type JMJD6 (JMJD6-wt) or catalytically inactive mutant JMJD6 (JMJD6-mut) or with the empty vector. Ectopically expressed JMJD6 protein was evaluated by Western blot analysis with an antiserum recognizing FLAG epitope and an antibody specific for JMJD6 (**Figure III-4E**). Consistent with the literature indicating that JMJD6 oligomerization requires JmjC domain catalytic activity (228), the wild type JMJD6 protein oligomerized, but the over-expressed mutant JMJD6 was only present as a monomer (**Figure III-4E**). The cells were subsequently transduced with the lentiviral vector containing shRNA to deplete endogenous JMJD6 protein. Upon adipogenic induction, a significant percentage of cells expressing FLAG-tagged wild type JMJD6 with endogenous JMJD6 depletion (JMJD6-wt/shJ6-3) differentiated into adipocytes (**Figure III-4F**). Interestingly, the cells expressing the catalytically inactive mutant JMJD6 (JMJD6-mut/shJ6-3) differentiated into adipocytes to the same extent as the cells expressing wild type JMJD6 (JMJD6-wt/shJ6-3) (**Figure III-4F**). Moreover, the protein levels of the reintroduced wild type and mutant JMJD6 proteins were similar to levels of endogenous JMJD6 in the control cells (**Figure III-4G, lane 4 and lane 6 compared to lane 1**). The expression levels of PPARγ

and C/EBPα protein were comparable in the wild type and in the mutant JMJD6 cells (**Figure III-4G, lane 4 and 6**). These results suggest that the catalytic activity of the JmjC domain in the JMJD6 protein is dispensable for the adipogenic differentiation of C3H10T1/2 cells.

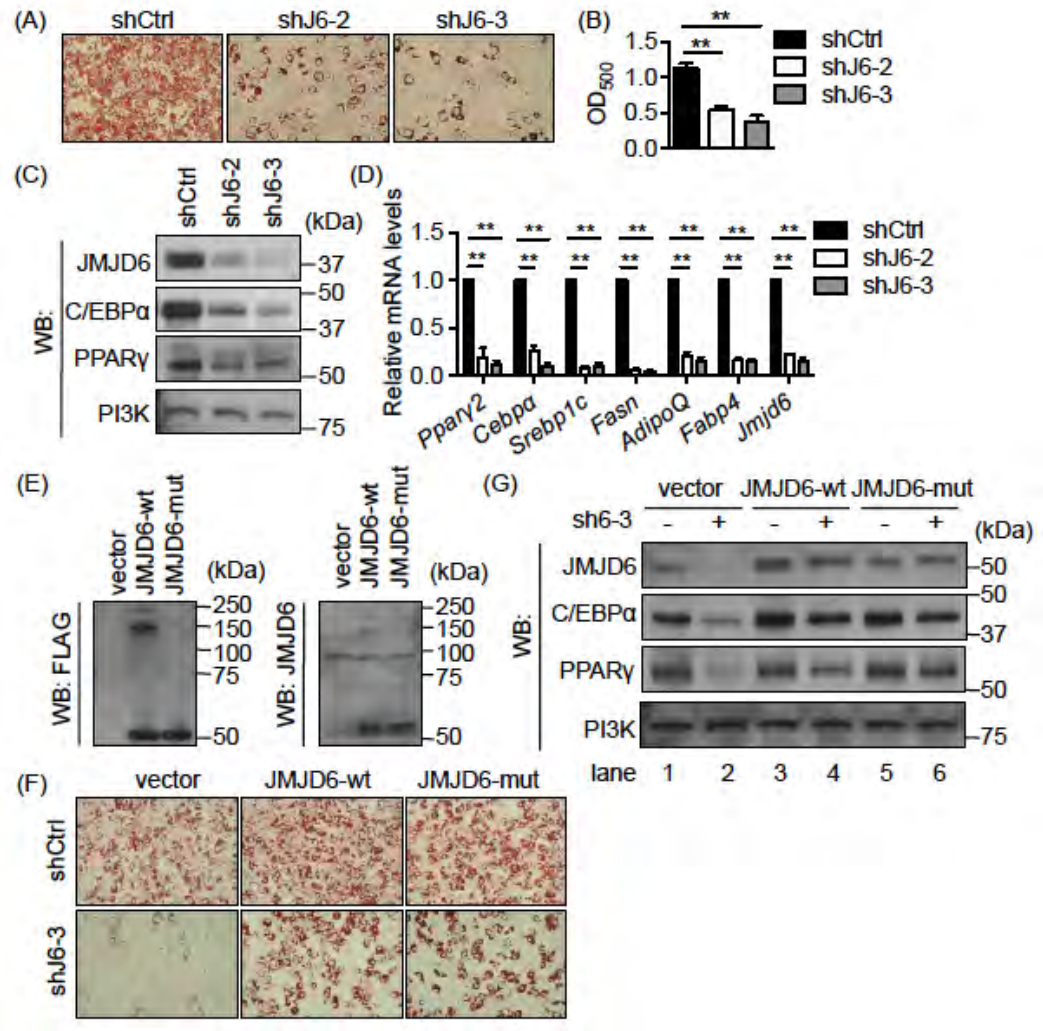

Figure III-4. JMJD6 is required for the adipogenic differentiation of C3H10T1/2 cells.
(A) Representative Oil-Red O staining images of day 6 post-induced C3H10T1/2 cells. Endogenous JMJD6 was knocked down by either of two shRNAs (shJ6-2 or shJ6-3). A scramble shRNA construct (shCtrl) was used a control. **(B)** Quantification of Oil Red O staining. The values are presented as the average of optical density at 500 nm (OD_{500}) from three independent experiments (S.E.M, n=3, ** p<0.01). **(C)** Representative western blots for

JMJD6, C/EBPα and PPARγ protein levels in the day 6 post-induced cells as indicated. PI3K levels were probed as a loading control. **(D)** The mRNA levels of adipocyte-associated genes in the day 6 post-differentiated cells. The individual mRNA levels were normalized to 5s rRNA levels. The normalized expression levels of the control cells were set as 1 (S.E.M, n=3, * p<0.05; ** p<0.01). **(E)** Representative western blots of the ectopically expressed wild type JMJD6 (JMJD6-wt) and catalytic-inactive mutant JMJD6 (JMJD6-mut) in C3H10T1/2 cells prior to differentiation. The blots were probed with either anti-FLAG antiserum or a JMJD6-specific antibody. **(F)** Representative Oil Red O staining images of day 6 post-differentiated cells as indicated. The C3H10T1/2 cells expressing either wild type JMJD6 (JMJD6-wt) or catalytic-inactive mutant (JMJD6-mut) or empty vector were subsequently transduced with lentiviral constructs containing either a scramble shRNA control (shCtrl) or a shRNA targeting the 3'UTR of *Jmjd6* mRNA (shJ6-3). **(G)** Representative western blots for JMJD6, C/EBPα and PPARγ protein levels in day 6 post-induced cells as indicated.

3.3.4 Knockdown of JMJD6 reduces C/EBPβ and C/EBPδ expression

To determine the causes of the differentiation defect in the JMJD6 knockdown cells, we analyzed the activation of *Ppar*γ and *Cebp*α genes, which is crucial for terminal differentiation. C/EBPβ and C/EBPδ are early adipogenic regulators that collaborate to activate *Ppar*γ and *Cebp*α transcription during the early stage of differentiation (183,191). By examining the expression of C/EBPβ and C/EBPδ, we found that JMJD6 knockdown resulted in a reduction of C/EBPβ and C/EBPδ protein expression at 3 h post-induction (**Figure III-5A**). However, we did not observe any effect of JMJD6 knockdown on *Cebp*β *and Cebp*δ mRNA levels (**Figure III-5B**), which suggests that JMJD6 is involved in the post-transcriptional control of C/EBPβ and C/EBPδ expression. Both *Cebp*β and *Cebp*δ are intronless genes. Therefore, the known function of JMJD6 in RNA splicing would not be a direct mechanism that caused the reduction C/EBPβ and C/EBPδ protein expression. We examined the protein stability of C/EBPβ and C/EBPδ by

cycloheximide treatment and Western blot analysis. Protein samples were collected for up to 5 hours in the presence of cycloheximide. No significant difference was detected in the degradation rate of C/EBPβ or C/EBPδ between the JMJD6 knockdown cells and the control cells (**Figure III-6**). Therefore, JMJD6 knockdown had no effect on C/EBPβ and C/EBPδ stability.

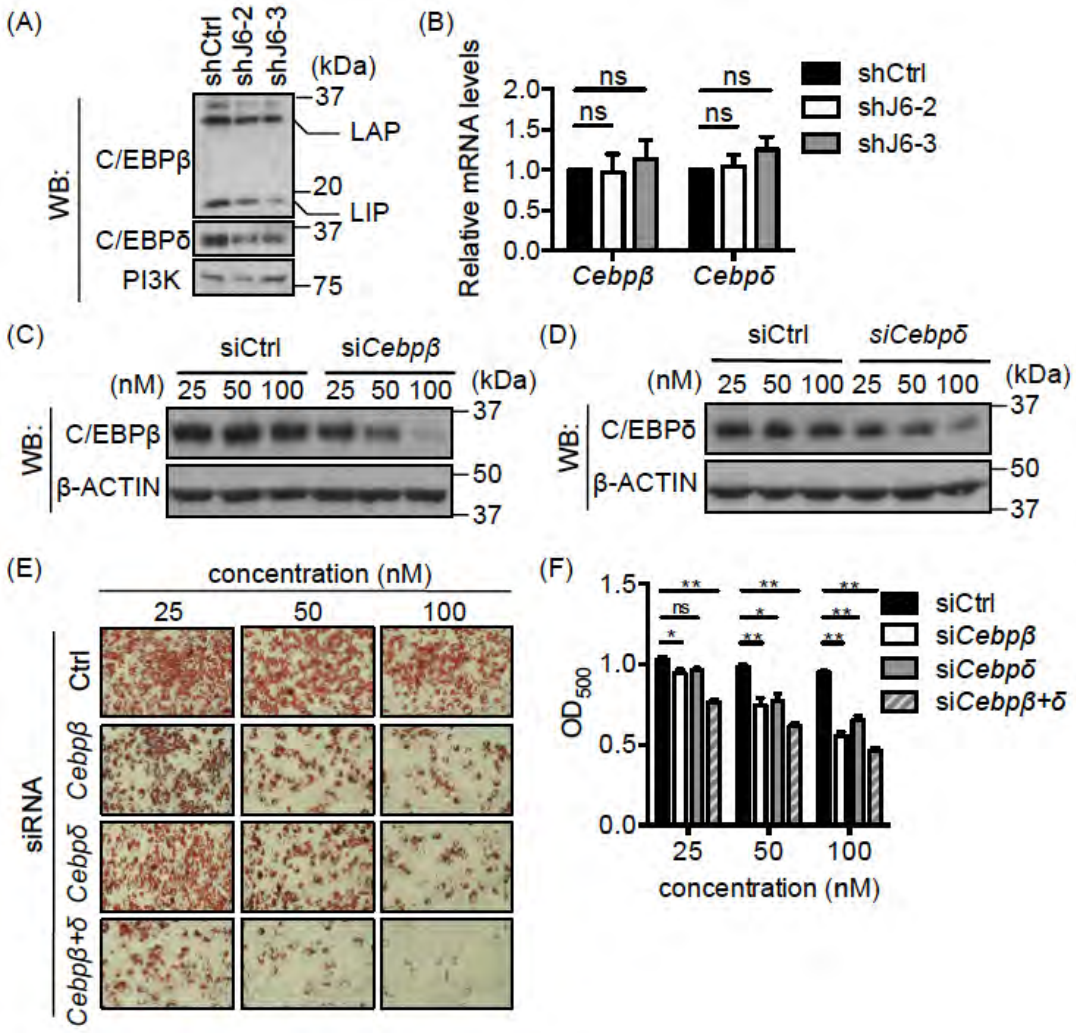

Figure III-5. Down-regulation of C/EBPβ and C/EBPδ by knockdown of JMJD6.

(A) Representative western blots for C/EBPβ and C/EBPδ protein levels in the 3 h post-induced control cells (shCtrl) and the JMJD6 knockdown cells (shJ6-2 and shJ6-3). PI3K levels were probed as a loading control. LAP and LIP represent different isoforms of C/EBPβ **(B)** *Cebpβ* and *Cebpδ*

mRNA levels in the 3 h post-induced cells. The normalized expression levels of the control cells were set as 1. The values are presented as the average of relative expression levels with standard error (S.E.M, n=3, ns: not significant). **(C-D)** Representative western blots for C/EBPβ and C/EBPδ protein levels in 3 h post-induced cells that were pre-treated with the indicated siRNAs for 24 h prior to adipogenic induction. β-ACTIN levels were probed as a loading control. **(E)** Representative Oil Red O staining images of day 6 post-induced C3H10T1/2 cells with siRNA treatment as indicated. **(F)** Quantification of Oil Red O staining. The values are presented as the average of optical density at 500 nm (OD_{500}) from three independent experiments (S.E.M, n=3, * p<0.05, ** p<0.01).

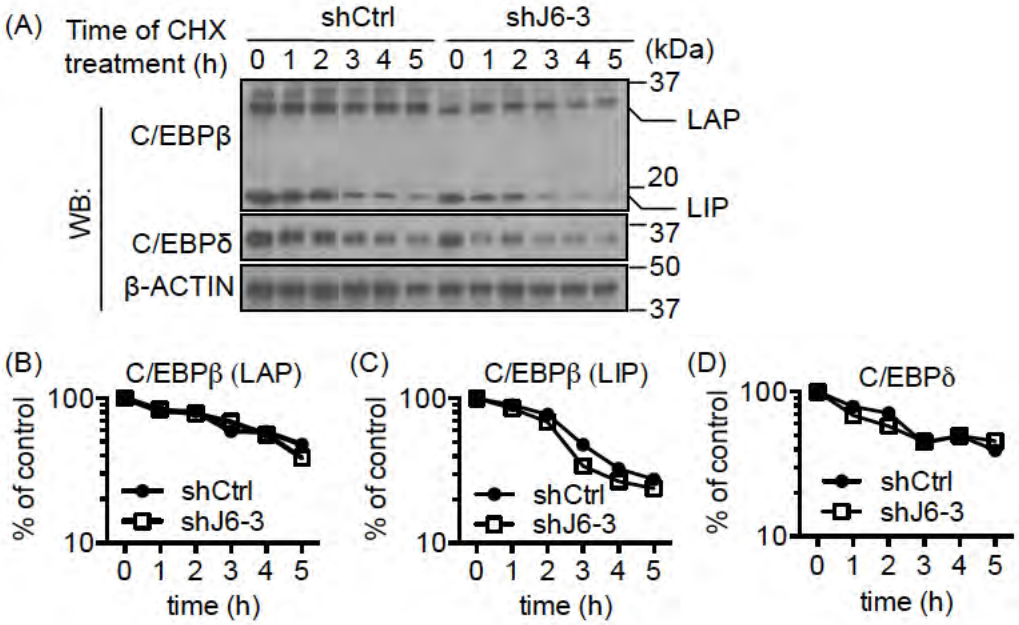

Figure III-6. Knockdown of JMJD6 has no effect on C/EBPβ and C/EBPδ protein stability.

(A) Representative western blots for C/EBPβ and C/EBPδ protein levels upon cycloheximide treatment. The 3 h post-induction cells were treated with 100 μg/ml cycloheximide. The total cell lysates were harvested every hour for 5 additional hours in the presence of cycloheximide. β-ACTIN was probed as a loading control. **(B-D)** C/EBPβ(LAP), C/EBPβ(LIP) and C/EBPδ protein levels in two independent experiments (n=2) were quantified by ImageJ. The protein levels were normalized to β-ACTIN loading control and were presented as the relative expression levels to the 0 h sample (mean, n=2).

Since JMJD6 knockdown did not completely abolish C/EBPβ and C/EBPδ expression, we examined whether suboptimal levels of C/EBPβ and C/EBPδ were sufficient to cause the differentiation deficiency. C3H10T1/2 cells were pre-treated with the siRNA targeting *Cebpβ* and *Cebpδ* mRNA 24 h prior to adipogenic induction. C/EBPβ and C/EBPδ protein levels were examined in 3 h post-induced cells (**Figure III-5C, D**). At a concentration of 50 nM, the siRNA treatment caused only a modest decrease in C/EBPβ and C/EBPδ protein levels. However, the adipogenic differentiation of C3H10T1/2 was significantly blocked (**Figure III-5E, F**). These results support the conclusion that the decease in C/EBPβ and C/EBPδ protein expression contributes to the differentiation deficiency in JMJD6 knockdown cells.

3.3.5 Knockdown of JMJD6 decreases C/EBPβ and RNA polymerase II binding at the *Pparγ2* and *Cebpα* gene loci

A previous study suggested that C/EBPβ functions as a pioneer transcription factor for the activation of the transcriptional program during the early stages of adipocyte differentiation (192). Since phosphorylation of C/EBPβ modulates its transcriptional activity (230), we further examined the effect of JMJD6 knockdown on the levels of C/EBPβ and phosphorylated C/EBPβ at threonine 188 in the differentiating cells. We found that JMJD6 knockdown reduced C/EBPβ levels throughout the first 48 h of differentiation (**Figure III-7A**), which is consistent with the previous results (**Figure III-5A**). Moreover, the level of C/EBPβ phosphorylation was directly proportional to the level of total C/EBPβ both

in shCtrl and shJ6-3 cells (**Figure III-7A and Figure III-8**), indicating that JMJD6 was not specifically affecting C/EBPβ phosphorylation of threonine 188. We therefore explored the possibility that reduced C/EBPβ protein levels might directly contribute to the activation of the *Pparγ2* and *Cebpα* genes by analyzing its binding to the corresponding promoter regions. As expected, ChIP assays showed that the binding of C/EBPβ was reduced at the *Pparγ2* and *Cebpα* proximal promoters in the JMJD6 knockdown cells upon adipogenic induction (**Figure III-7B, C**), which correlates with the inhibition of *Pparγ2* and *Cebpα* expression. It has been shown that JMJD6 directly contributes to RNA polymerase II transcription through binding to distal enhancers (142). We therefore examined the binding of JMJD6 and RNA polymerase II at the *Pparγ2* and *Cebpα* loci and their putative enhancers. For calling the putative enhancers, we searched published datasets for genomic regions that were enriched with DNAse I hypersensitivity (DHS), MED1, p300, and BRG1 binding, and H3K4me1/2 and H3K27Ac incorporation but with low levels of H3K4me3 incorporation (**Figure III-9**) (201,202). The -9.5kb, +96kb, and +100kb regions of the *Pparγ2* locus and +37kb regions of the *Cepbα* were previously called as putative enhancers in immortalized brown preadipocytes (201). ChIP-qPCR results showed that the binding of JMJD6 increased upon differentiation across the *Pparγ2* and *Cebpα* genomic loci (**Figure III-10A, B**). In addition, the binding of JMJD6 and RNA polymerase II at most of the sequences examined was decreased in the JMJD6 knockdown cells (**Figure III-7D to G**). These data suggest that the failure of *Pparγ2* and *Cebpα* gene activation caused

by the JMJD6 knockdown is likely a consequence of the reduced binding of C/EBPβ, JMJD6, and RNA polymerase II at these genomic loci.

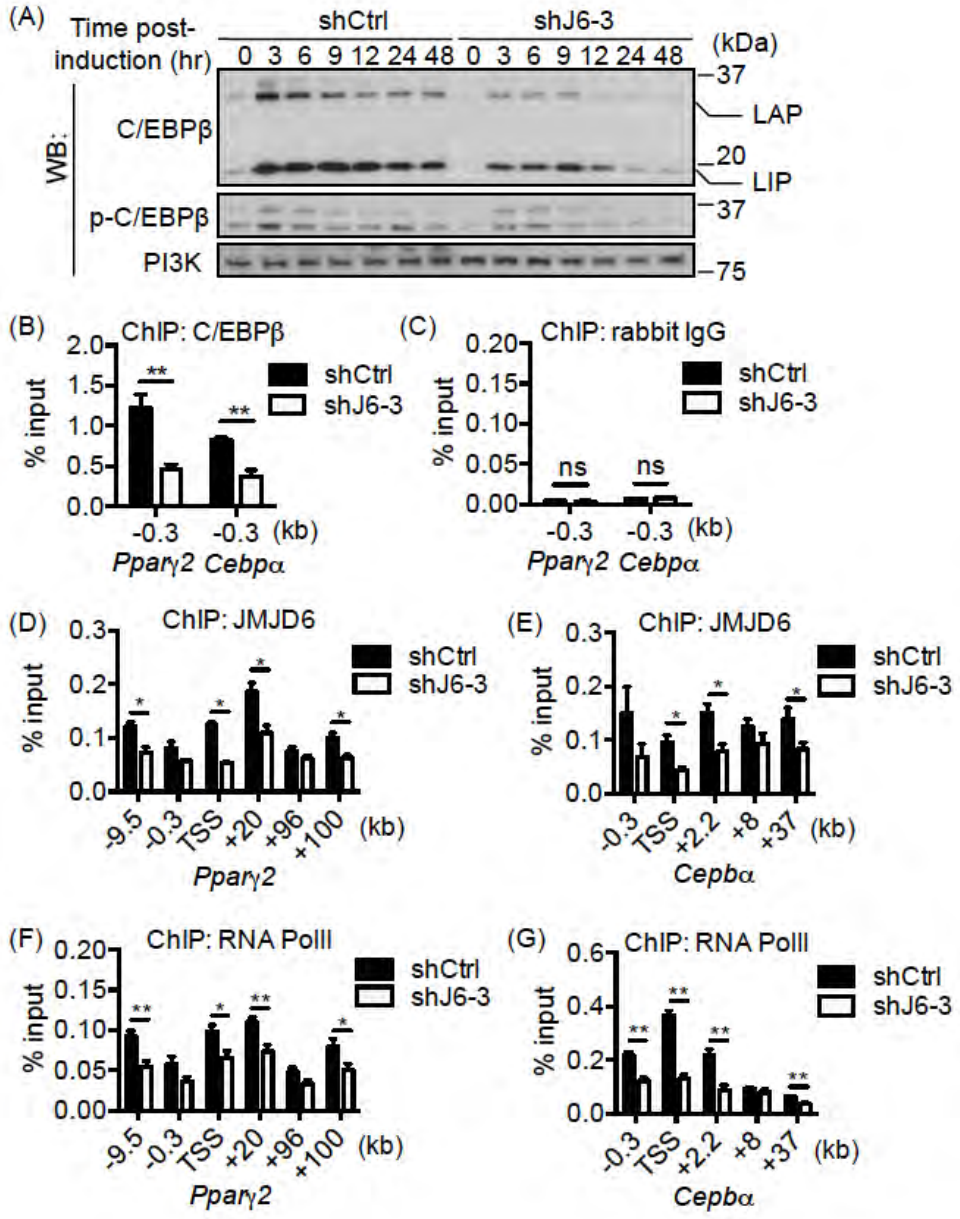

Figure III-7 Knockdown of JMJD6 reduced C/EBPβ and RNA polymerase II occupancy at the *Pparγ2* and *Cebpα* loci.
(A) Representative western blots for the levels of C/EBPβ and phosphorylated C/EBPβ at threonine 188 in the control cells (shCtrl) and in the JMJD6 knockdown cells (shJ6-3) throughout the early phase of differentiation. PI3K levels were probed as a loading control. **(B)** ChIP of

C/EBPβ at the *Pparγ2* and *Cebpα* proximal promoters. **(C)** ChIP of rabbit IgG for negative control. **(D-E)** ChIP of JMJD6 and **(F-G)** ChIP of RNA polymerase II at the *Pparγ2* and *Cebpα* loci and nearby putative enhancers. The values are presented as the average % input from at least three independent experiments (S.E.M, n≥3, * $p<0.05$, ** $p<0.01$).

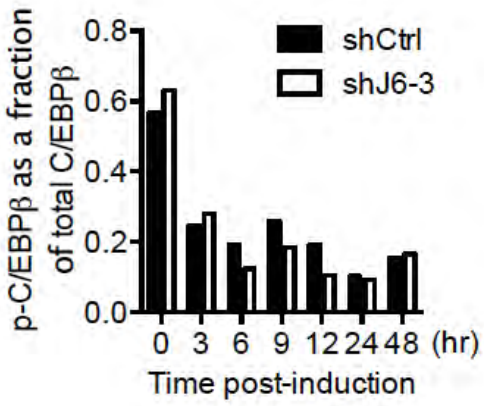

Figure III-8 The phosphorylated C/EBPβ is proportional to the total C/EBPβ in both control and JMJD6 knockdown cells.
The levels of C/EBPβ and phosphorylated C/EBPβ in Figure III-7A were quantified by ImageJ.

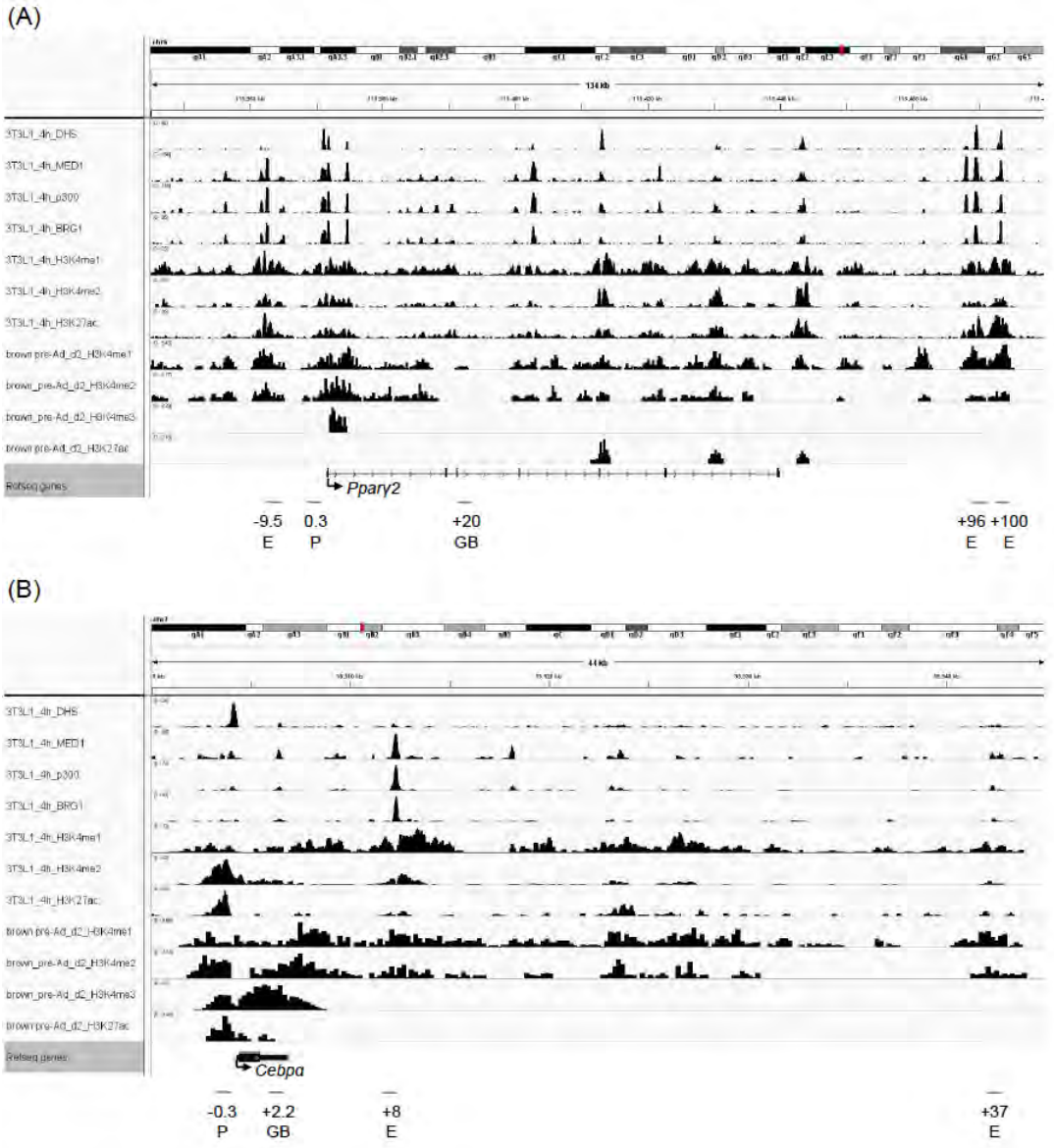

Figure III-9. Calling the putative enhancers using the published DHS-seq and ChIP-seq datasets.

The DHS-seq datasets (GSE27826) and ChIP-seq datasets (GSE56872 and GSE50466) were uploaded to Integrative Genomics Viewer. (A) Screenshot of *Pparγ2* locus. (B) Screenshot of *Cebpα* locus. Blue bars indicate the regions for primer design. P: promoter; E: putative enhancer; GB: gene body.

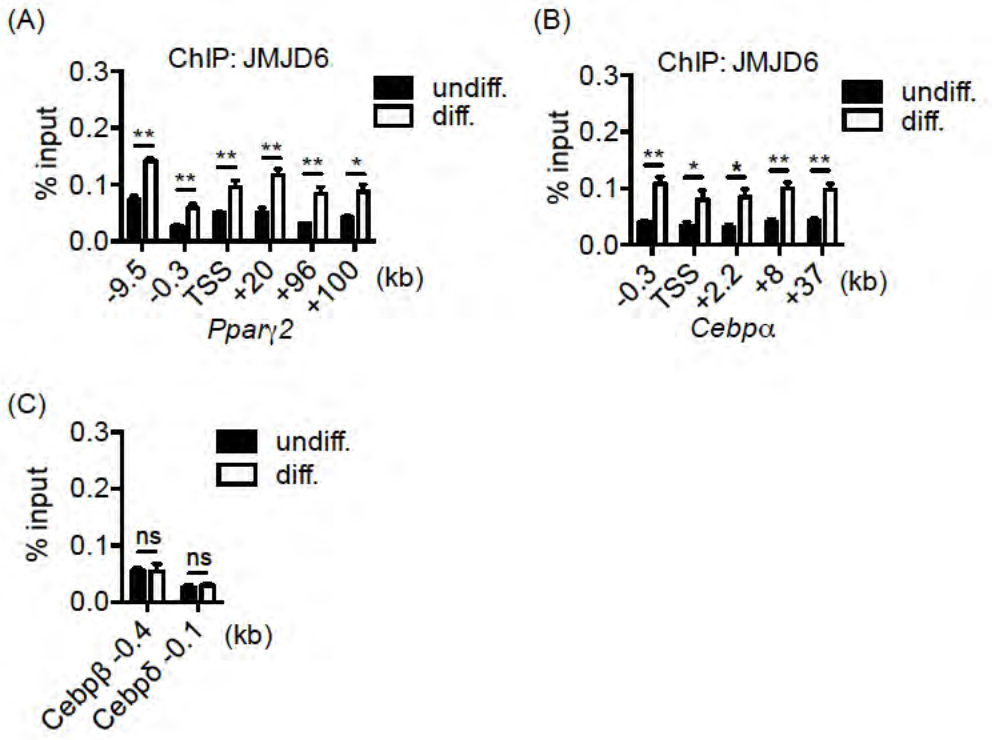

Figure III-10. Inducible binding of JMJD6 at the *Pparγ2* and *Cebpα* loci during differentiation.
(A-B) ChIP of JMJD6 at the *Pparγ2* and *Cebpα* loci in the undifferentiated (0h post-induced) and in the differentiating (24h post-induced) C3H10T1/2 cells. The values are presented as the average % input from three independent experiments (S.E.M, n=3, **p*<0.05, ***p*<0.01). **(C)** The binding of JMJD6 at the *Cebpβ* and *Cebpδ* promoters was examined as negative controls. The values are presented as the average % input from three independent experiments (S.E.M, n=3, ns: not significant)

3.3.6 Ectopic expression of C/EBPβ and C/EBPδ is not sufficient to rescue the differentiation deficiency

To determine whether the JMJD6-dependent defects in *Pparγ2* and *Cebpα* gene activation were entirely related to the decrease in C/EBPβ and C/EBPδ protein levels, we attempted to rescue the adipogenic differentiation in the JMJD6 knockdown cells by ectopically expressing C/EBPβ and C/EBPδ. Transient

transfection of the plasmids encoding C/EBPβ (LAP isoform) and C/EBPδ protein into the scramble control cells resulted in robust expression of C/EBPβ and C/EBPδ (**Figure III-11A**). However, the JMJD6 knockdown cells failed to express these proteins (**Figure III-11A**), even though the plasmid-derived mRNAs were present in the JMJD6 knockdown cells (**Figure III-12A, B**). These data suggest that the post-transcriptional mechanism by which JMJD6 regulates endogenous C/EBPβ and C/EBPδ also regulates the ectopic expression of these proteins. To bypass the post-transcriptional defects resulting from the knockdown of JMJD6, we introduced *Cebpβ* and *Cebpδ* mRNAs that were synthesized *in vitro* (**Figure III-12C, D**). This procedure generated equivalent protein expression both in the JMJD6 knockdown cells and in the control cells (**Figure III-11B**). We then sought to determine whether the introduction of *Cebpβ* and *Cebpδ* mRNA could rescue the differentiation deficiency resulting from JMJD6 knockdown. For the mRNA transfection control, we chose *in vitro* transcribed EGFP mRNA because of the length similarity to *Cebpβ* and *Cebpδ* mRNA and the minimal effect of EGFP on the transcriptional program. As shown by Oil Red O staining, ectopic expression of *Cebpβ* and/or *Cebpδ* mRNA was not sufficient to rescue the differentiation deficiency (**Figure III-11C**). The JMJD6 knockdown cells transfected with a combination of *Cebpβ* and *Cebpδ* mRNA failed to express endogenous *Pparγ2* and *Cebpα* mRNA to the levels observed in the control cells (**Figure III-11D**). Based on these results, we conclude that the JMJD6 knockdown cells must have multiple defects in *Pparγ*2 and *Cepbα* gene activation. Since JMJD6 was present at the *Pparγ*2 and *Cepbα* genomic loci (**Figure III-7D, E**), we speculated that

JMJD6 might directly contribute to the transcriptional activation of *Pparγ2* and *Cepbα.*

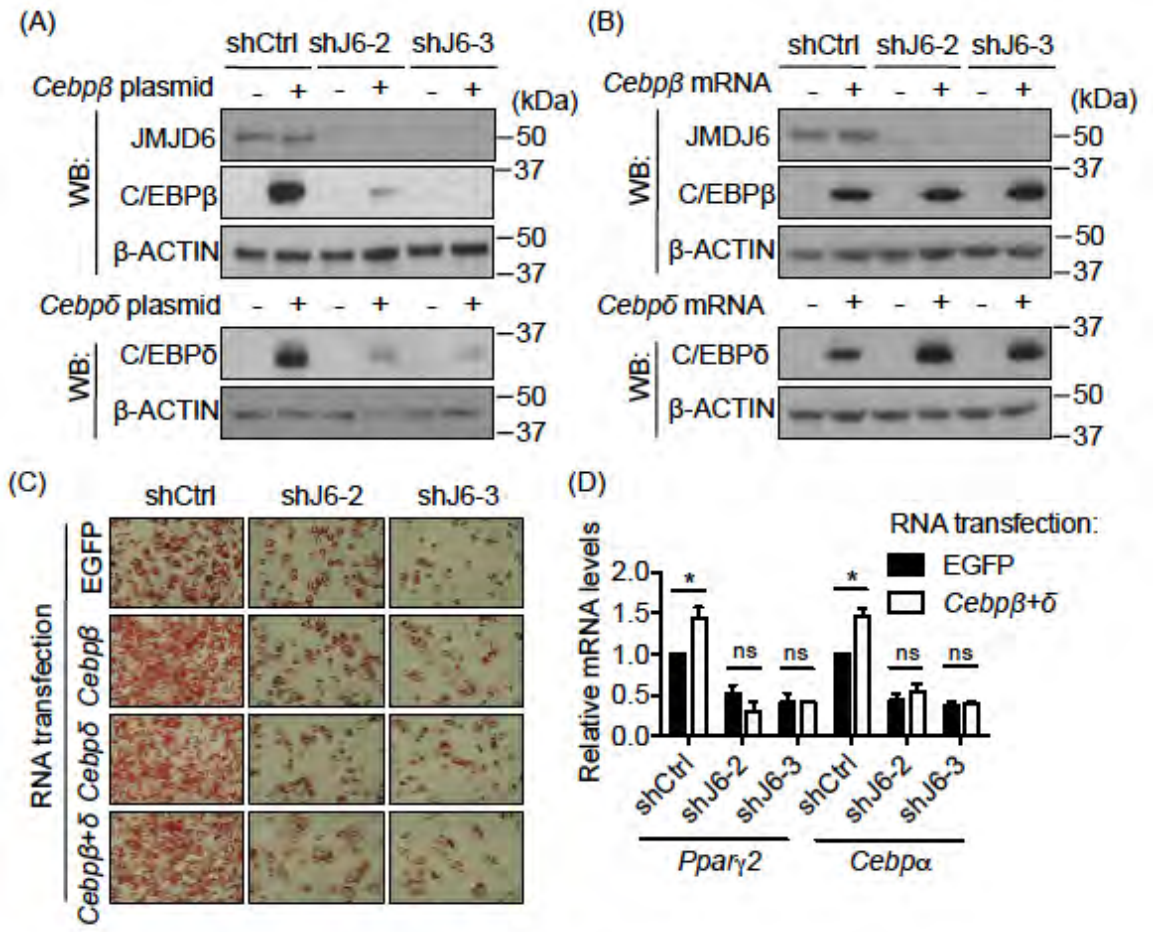

Figure III-11. Ectopic expression of C/EBPβ and C/EBPδ is not sufficient to rescue the differentiation deficiency.
(A) Representative western blots for the levels of C/EBPβ, C/EBPδ and JMJD6 protein expression in the control cells (shCtrl) and in the JMJD6 knockdown cells (shJ6-2 and shJ6-3) 24 h after transfection with the plasmids encoding C/EBPβ(LAP) and C/EBPδ protein. An empty vector was used as a transfection control. **(B)** C/EBPβ, C/EBPδ and JMJD6 protein expression in the cells 24 h after transfection with *in vitro* synthesized *Cebpβ* and *Cebpδ* mRNA. *In vitro* synthesized EGFP mRNA was used as a transfection control. **(C)** Representative Oil Red O staining images of the day 6 post-induced cells as indicated. **(D)** *Pparγ2* and *Cebpα* mRNA levels in the day 6 post-induced cells that were transfected with EGFP mRNA or a combination of *Cebpβ* and *Cebpδ* mRNA (*Cebpβ+δ*) as indicated. The

values are presented as the average of relative expression levels with standard error (S.E.M, n=3, * p<0.05; ** p<0.01, ns: not significant).

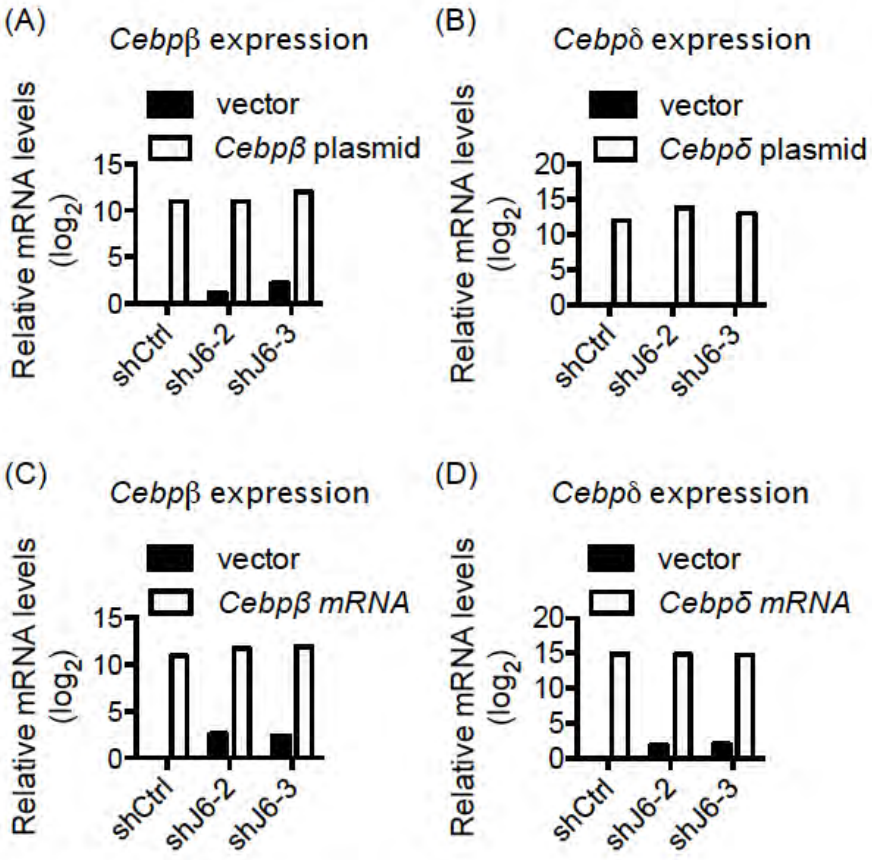

Figure III-12. Ectopic expression of C/EBPβ and C/EBPδ by introduction of plasmids or *in vitro* synthesized RNAs.
(A-B) *Cebpβ* and *Cebpδ* mRNA levels in the plasmid-transfected cells. The cells were transfected with the plasmids encoding either C/EBPβ or C/EBPδ or empty vector for 24 h and then harvested for gene expression analysis. (**C-D)** *Cebpβ* and *Cebpδ* mRNA levels in the RNA-transfected cells. The cells were transfected with the *in vitro* synthesis mRNA encoding either C/EBPβ or C/EBPδ for 24 h and harvested for gene expression analysis. The values presented are the average relative expression levels from technical replicates of the matching RNA samples from the western blot experiment presented in Figure. III-11 A-B.

3.3.7 The BET protein inhibitor JQ1 inhibits the transcriptional program for adipocyte differentiation

JMJD6 has no characterized DNA binding domain (231) and also lacks DNA binding ability *in vitro* (160). Studies have shown that JMJD6 physically interacts with the chromatin reader proteins BRD4 and BRD2 (142,232). To determine the mechanisms by which JMJD6 binds to the genomic loci and directly controls *Pparγ2* and *Cepbα* gene activation, we targeted the chromatin reader for JMJD6 by the BET protein-specific inhibitor JQ1 (233). We found that JQ1 treatment significantly inhibited adipogenic differentiation at a 1 μM dose (**Figure III-13A, B**). By analyzing the expression of adipogenic regulators, we found that JQ1 treatment blocked PPARγ and C/EBPα protein expression (**Figure III-13C**). Unlike in the JMJD6 knockdown cells, JQ1 had no effect on C/EBPβ and C/EBPδ levels at either 3 or 24 hours post-induction (**Figure III-13C**). Consistent with the protein analysis, JQ1 treatment inhibited the expression of *Pparγ2* and *Cebpα* mRNAs without or with minimal effect on *Cebpβ* and *Cebpδ* mRNA expression, respectively (**Figure III-13D**). The data indicate that the inhibition of adipogenic differentiation by JQ1 occurs via a mechanism that affects PPARγ2 and C/EBPα expression without affecting expression of the C/EBPβ and C/EBPδ. Though JQ1 affects neither the expression of JMJD6 (**Figure III-13C**) nor the interaction of JMJD6 with BRD4 (**Figure III-14**), ChIP experiments showed that JQ1 treatment reduced the binding of JMJD6, BRD4, and RNA polymerase II at the *Pparγ2* and *Cepbα* loci and their putative enhancers (**Figure III-15A to F**), thereby providing a mechanism for the inhibition of *Pparγ2* and *Cebpα* gene expression. JQ1

treatment did not affect the binding of C/EBPβ and C/EBPδ at the *Pparγ2* and *Cebpα* promoter regions (**Figure III-15G**, **H**). These results indicate that BRD4-mediated JMJD6 chromatin binding at the *Pparγ2* and *Cebpα* genomic loci contributes to gene activation during differentiation.

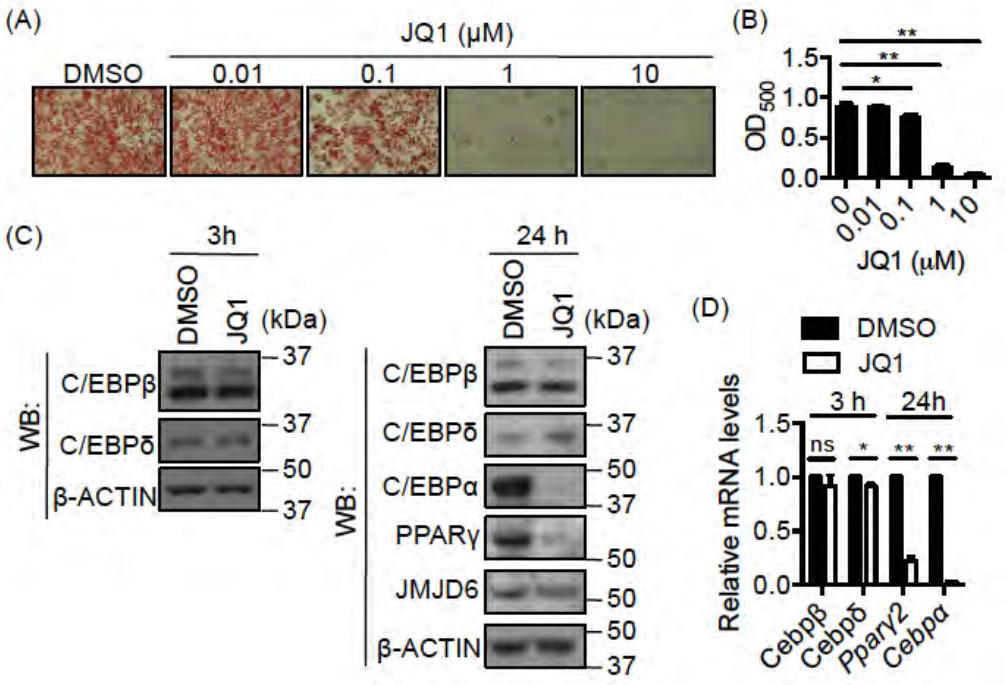

Figure III-13. The BET protein inhibitor JQ1 inhibits the adipogenic differentiation of C3H10T1/2 cells.
(A) Representative Oil Red O staining images of day 6 post-induced C3H10T1/2 cells treated with JQ1 or DMSO. The treatment was performed in the first 48 hours during the adipogenic induction. **(B)** Quantification of Oil Red O staining. The values are presented as the average of optical density at 500 nm (OD_{500}) from three independent experiments (S.E.M, n=3, * p<0.05, ** p<0.01). **(C)** Representative western blots for the indicated proteins in the 3 h and 24 h post-induced cells in the presence of 1 μM JQ1 or DMSO as a vehicle control. **(D)** The mRNA levels of the indicated genes in the 3 h and 24 h post-induced cells in the presence of 1 μM JQ1 or DMSO. The values are presented as the average of relative expression levels from three independent experiments with standard error (S.E.M, n=3, * p<0.05; ** p<0.01, ns: not significant).

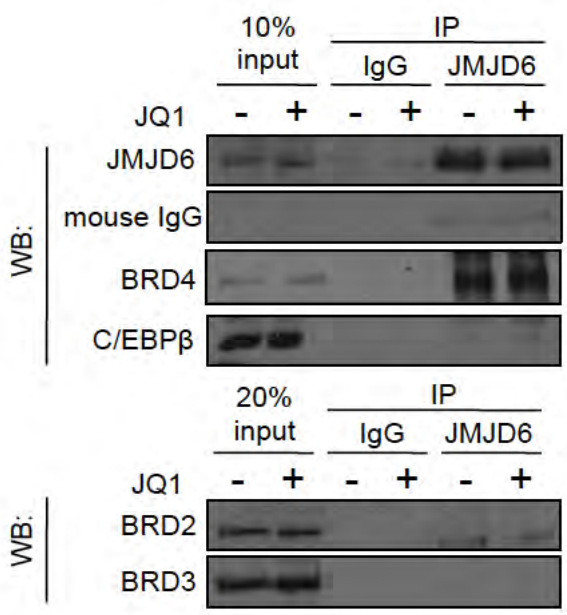

Figure III-14. BRD4 is immunoprecipitated with JMJD6.
Endogenous JMJD6 proteins in day1 post-induced C3H10T1/2 cells with and without JQ1 treatment were immunoprecipitated. The eluted samples were run on 8% SDS-PAGE for western blotting. The blots were probed with antibodies against JMJD6 (sc-28349), mouse IgG (NA9310), BRD4 (A301-985A50), C/EBPβ (sc-150), BRD2 (A302-583A), and BRD3 (A302-367A).

Collectively, the data support the conclusion that JMDJ6 acts as both a post-transcriptional and a transcriptional regulator during adipogenesis. A schematic diagram summarizing our findings is presented in **Figure III-16**.

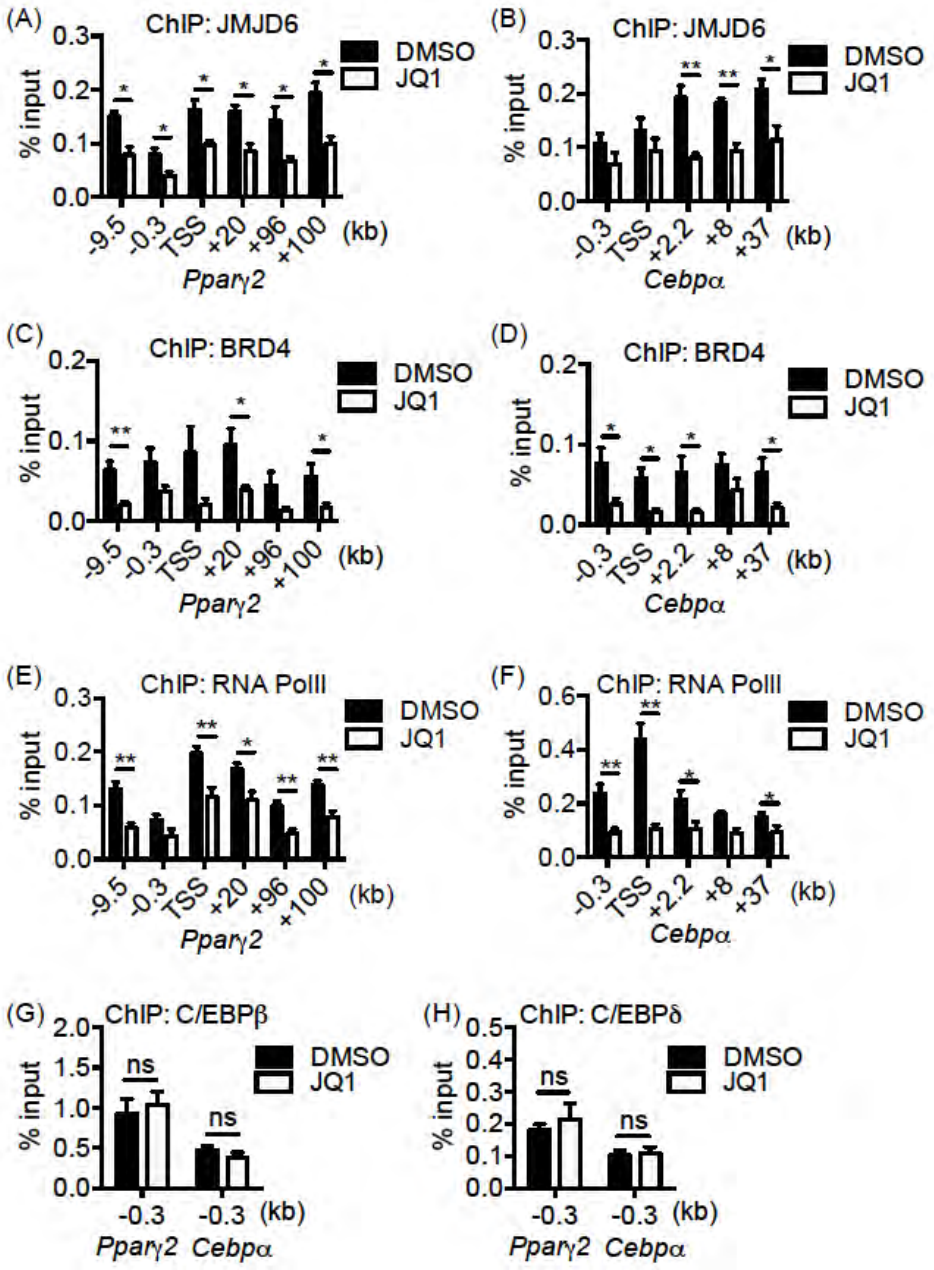

Figure III-15. JQ1 treatment reduces the binding of JMJD6 and RNA polymerase II at the *Pparγ2* and *Cebpα* loci.

(A-B) ChIP of JMJD6, **(C-D)** ChIP of BRD4, and **(E-F)** ChIP of RNA polymerase II at the *Pparγ2* and *Cebpα* loci and nearby putative enhancers in 24 h post-induced C3H10T1/2 cells in the presence of 1μM JQ1 or DMSO. **(G)** ChIP of C/EBPβ and **(H)** ChIP of C/EBPδ at the *Pparγ2* and *Cebpα* proximal promoters in 24 h post-induced C3H10T1/2 cells. The values are presented as the average % input from four independent experiments (S.E.M, n = 4, * p<0.05, ** p<0.01, ns: not significant). For

negative control, ChIP of rabbit IgG was performed under the same conditions. The average % input from rabbit IgG ChIPs was below 0.05% in all cases (data not shown).

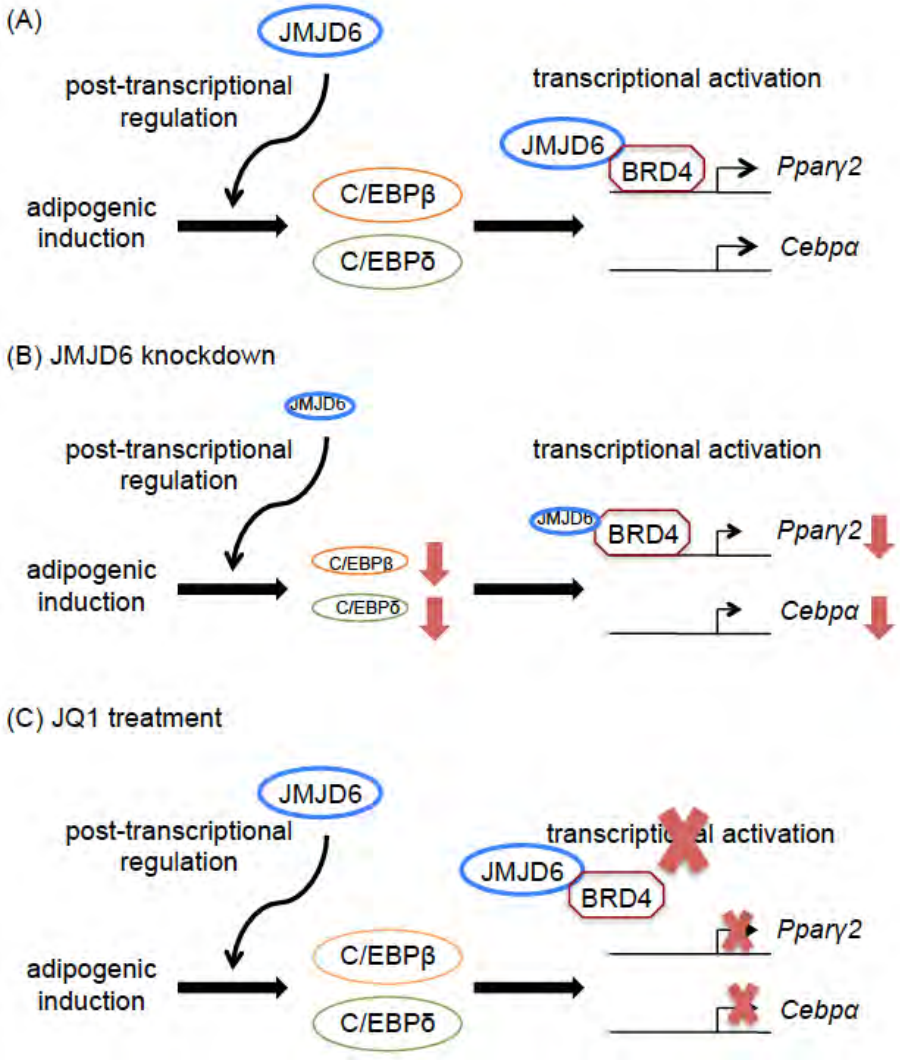

Figure III-16. The dual functions of JMJD6 in control of adipogenic gene expression.
(A) JMJD6 participates in the post-transcriptional regulation of C/EBPβ and C/EBPδ and transcriptional activation of *Pparγ2* and *Cebpα* genes. **(B)** Upon JMJD6 knockdown, dual functions of JMJD6 are compromised, and that results in a reduction of C/EBPβ and C/EBPδ protein levels and the mRNA levels from the *Pparγ2* and *Cebpα* genes. **(C)** Treatment of JQ1 blocks the transcriptional activation function of JMJD6 without an effect on C/EBPβ and C/EBPδ protein levels.

3.4 Discussion

The effect of JMJD6 knockdown on C/EBPβ and C/EBPδ expression suggests a novel role of JMJD6 in the post-translational control of gene expression. We had determined that JMJD6 knockdown had no effect on C/EBPβ and C/EBPδ protein stability, and since both *Cebpβ* and *Cebpδ* are intronless genes, the reduction of C/EBPβ and C/EBPδ expression cannot be due to a defect in splicing of these two transcripts. Moreover, the general translation machinery is unlikely affected, because the *in vitro* synthesized *Cebpβ* and *Cebpδ* mRNA can be translated in the JMJD6 knockdown cells. Therefore, we concluded that JMJD6 promotes C/EBPβ and C/EBPδ expression at one or more steps between RNA synthesis and translation. The known JMJD6 interacting proteins are mostly involved in RNA processing (151). In addition, structural and biochemical characterization of JMJD6 also suggests a broad RNA binding capability (160). Perhaps JMJD6 is a part of *Cebpβ* and *Cebpδ* messenger ribonucleoprotein particles (mRNPs) and is involved in the assembly, processing, remodeling and localization of these mRNPs. Alternatively, JMJD6 might facilitate mRNA binding to ribosomes, which would be supported by the known interaction with ribosomal proteins and translation initiation factors (151,229). Taken together, we propose that JMJD6 plays multiple roles in post-transcriptional regulation of gene expression, regulating splicing on intron containing genes and as well as a second function related to other aspects of RNA processing. To get insight on how JMJD6 is involved in the various steps of mRNA metabolism in the context of adipogenesis, it would be interesting to identify and characterize the interacting

protein partners as well as the associated RNA species with unbiased approaches such as immunoprecipitation followed by mass spectrometry or deep sequencing.

Though our studies demonstrated that JMJD6 dependent regulation of C/EBPβ and C/EBPδ expression contributes to the activation of the lineage determinants *Pparγ2* and *Cebpα*, the work also indicates that this function is not sufficient to drive *Pparγ2* and *Cebpα* expression and adipogenic differentiation. Thus there are additional roles for JMJD6 in promoting adipogenesis. Our ChIP results showed that JQ1 treatment caused the loss of JMJD6 binding at the *Pparγ2* and *Cebpα* loci in a manner independent of controlling C/EBPβ and C/EBPδ expression. Therefore, JMJD6 likely directly contributes to the transcriptional activation of *Pparγ2* and *Cebpα* genes, perhaps in cooperation with the known JMJD6 interacting protein, BRD4. Consistent with this hypothesis, treatment of cells with JQ1 caused a decrease in BRD4 and RNA polymerase II occupancy at the examined loci. Though previous work by others connected JMJD6 to the release of RNA polymerase II pausing (142), it appears that in the context of *Pparγ2* and *Cebpα* activation, JMJD6 may also promote RNA polymerase II recruitment. The decrease of RNA polymerase II binding at the putative enhancers near the *Pparγ2* and *Cebpα* loci indicates that both JMJD6 knockdown and JQ1 treatment might not only inhibit the transcription of *Pparγ2* and *Cebpα* genes but also the enhancer-derived RNAs (eRNAs). Noncoding eRNA transcripts have been indentified in a number of cell types including adipocytes (203,234). Although the function of eRNAs are not fully understood, several lines of evidence suggest

the roles of eRNA in promoting enhancer-promoter chromatin looping (235) and RNA polymerase II recruitment at promoters and gene bodies (236,237). Therefore the contributions of JMJD6 to transcription might occur via both promoter and enhancer sequences.

Studies examining the effects of JQ1 on RNA polymerase II transcription have indicated that JQ1 selectively binds to the BRD4 bromodomain, and prevents the genomic binding by BRD4 and its associated factors, pTEFb and Mediator (238,239). Our study indicates that JMJD6 is one of the BRD4-associated proteins in the differentiating cells (**Figure III-14**), and the binding of JMJD6 at the genomic loci is prevented by JQ1 (**Figure III-15**). However, the inhibitory effects of JQ1 on the adipogenic transcription program might be pleiotrophic, because BRD4 interacts with multiple transcription factors and chromatin regulators in addition to acetylated histones through its bromodomain (240). Furthermore, it is important to note that JQ1 has high affinity for the bromodomains of BRD2, BRD3, BRD4 and BRDT (233). Except for testes/oocyte-specific BRDT, BRD2, BRD3, BRD4 might all contribute the gene transcription program during adipogenic differentiation, possibly because these BET proteins facilitate transcriptional elongation through acetylated nucleosomes (241,242). Further complicating matters, BRD2 shows anti-adipogenic function in a hypomorphic mouse model as well as in 3T3-L1 preadipocytes (243,244). It is still unclear how BRD2 represses *Pparγ2* gene expression, but the evidence indicated that BRD2 associates with PPARγ and represses the ability of PPARγ to activate downstream target genes (243). There

may therefore be a regulatory balance between positive and negatively acting BET proteins. Although we did not indentify any interaction of JMJD6 with BRD2 and BRD3 at the time point we assayed in the differentiating cells by co-immunoprecipitation (**Figure III-14**), since JMJD6 potentially interacts with both BRD2 and BRD4 (142,232), it is possible that JMJD6 collaborates with these distinct chromatin readers in a temporal-and spatial-dependent manner that differentially regulates the adipogenic transcriptional program.

JMJD6 was originally identified as an arginine demethylase (141) but subsequent work from different groups has been contradictory; some studies support the original findings while others do not and suggest that JMJD6 instead acts as a lysine hydroxylase (142,144,151,157,228,245). Our study demonstrated that the adipogenic function of JMJD6 is independent of JmjC domain activity. A recent study also reported a JmjC domain independent function of JMJD6 in the splicing of a reporter gene (159). Thus while JMJD6 has the potential to post-translationally modify chromatin and/or proteins involved in transcription, splicing, or any other cellular process, any such activity is not required for adipogenic differentiation, or, at best, contributes to the process in a non-essential manner. Given the diverse roles described for JMJD6, perhaps it functions as a scaffold protein that facilitates the localization and functions of other regulatory proteins.

The idea of JMJD6 function that is independent of JmjC domain catalytic activity raises the question of what JMJD6 domains mediate its functions. In

addition to the JmjC domain, JMJD6 has a polyserine domain, multiple nuclear localization sequences, a nuclear export sequences, an AT-hook DNA binding motif, and a sumoylation site (231,246). To date, only the polyserine domain and nuclear localization sequences have been functionally evaluated (229,246,247). We attempted to identify regions of JMJD6 that are necessary for adipogenic differentiation by ectopically expressing a series of truncated JMJD6 proteins in C3H10T1/2 cells. However, the expression levels of these truncated JMJD6 proteins were either very low or not detectable (**Figure III-17**). These findings are consistent with the work of others who have also reported that truncations and deletions in JMJD6 decrease protein stability or form protein aggregates when they are ectopically expressed (227). Therefore, the domain(s) of JMJD6 necessary for the adipogenic function is still unclear. A systematic structure-function study using point mutations or small deletions would be necessary to identify the JmjC domain-independent functions of JMJD6.

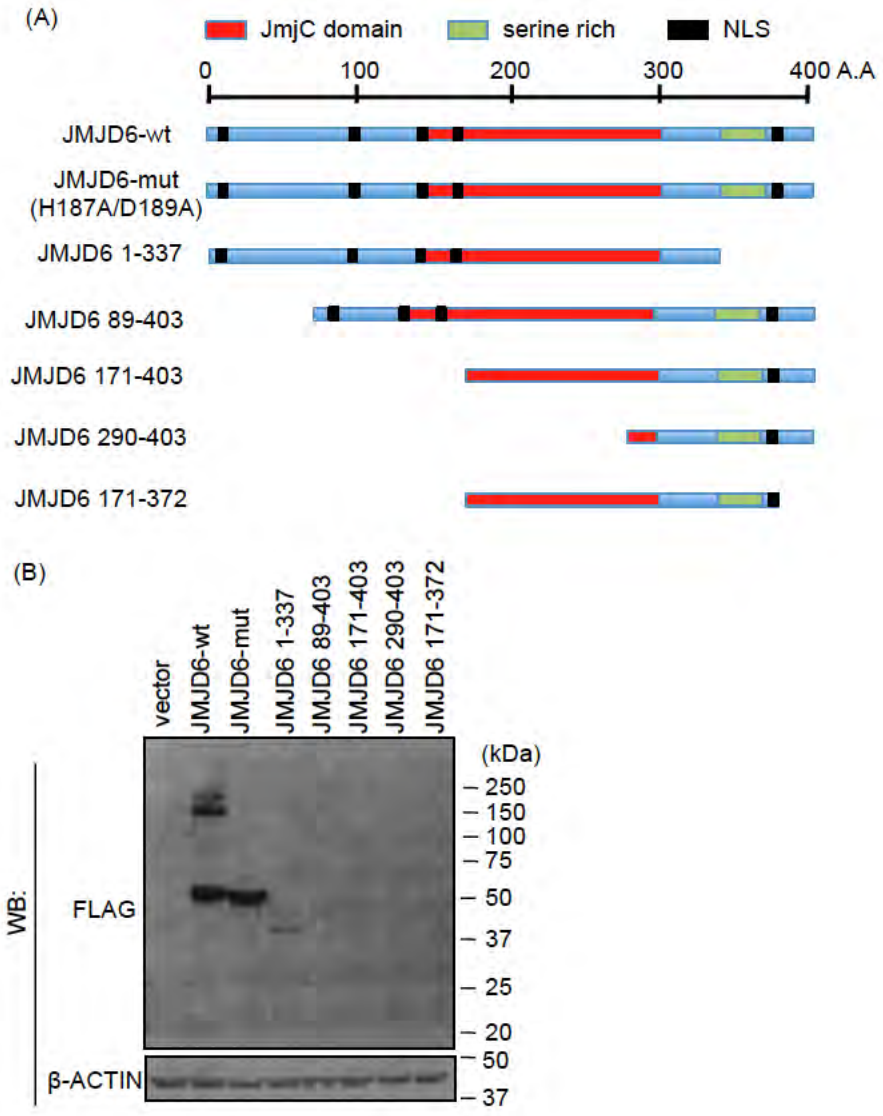

Figure III-17. Ectopic expression of JMJD6 proteins in C3H10T1/2 cells.

(A) Scheme of the full-length wild type, catalytic-inactive and truncated JMJD6 mutants. The DNA sequences encoding the truncated proteins were PCR amplified and cloned into a pBABE vector as the same as for the wild type and catalytic-inactive JMJD6 mutant. **(B)** A representative western blot for the expression of JMJD6 proteins in C3H10T1/2 cells. The blot was probed with a serum recognizing FLAG sequence and re-probed with an antibody for β-ACTIN as a loading control.

CHAPTER IV

Discussion and Conclusions

4.1 Role of PRMTs in adipocyte differentiation and function

In the context of adipocyte differentiation, PRMT5 and PRMT4 are transcriptional co-activators for PPARγ and PPARγ target genes (55,79). The results presented in this thesis suggest that PRMT7 is not essential for the differentiation of adipocytes. The role of other PRMTs in adipocyte differentiation remains to be elucidated. PRMT2, PRMT3, PRMT6 deficient mice are viable with no developmental defects (49-51,59) and PRMT8 is expressed specifically in brain (17). Therefore, these PRMTs are unlikely to affect adipogenic differentiation.

PRMT1 might play a role in adipocyte differentiation, though the direct connection between PRMT1 and differentiation has not been examined experimentally. The transcription factor FOXO1 is a negative regulator for adipocyte differentiation (248). Exposure of preadipocyte to insulin induces Akt-dependent phosphorylation of FOXO1 and its nuclear export. Methylation of FOXO1 by PRMT1 prevents the Akt-dependent phosphorylation, thereby increases its nuclear accumulation and function (101), which is known to prevent adipocyte differentiation. PRMT1 might also have a role in the formation of brown adipocytes, because PRMT1 methylates transcriptional co-regulator PGC-1α (110) and RIP140 (111,249). PGC-1α is a PPARγ-associated transcriptional co-activator that activates the genes involved in mitochondrial biogenesis and adaptive

thermogenesis (250). RIP140 acts a co-repressor that represses the genes involved in energy expenditure (251-253). Methylation of PGC-1α by PRMT1 stimulates its co-activator activity(110), whereas methylation of RIP140 suppresses its co-repressor function (111,249). Therefore, PRMT1 might promote brown adipocyte formation or white adipocytes browning by methylating PGC-1α and RIP140.

The biological roles of PRMTs in post-differentiated adipocytes are largely unknown. Metabolic diseases, including obesity and diabetes, are tightly associated with adipocyte dysfunctions such as changes in lipid storage and mobilization, insulin sensitivity, cytokine and peptide hormone production (254). The limited efficiency of plasmid transfection and viral transduction in mature adipocytes makes it difficult to perform gain-of-function or loss-of-function study. In order to examine the roles of PRMTs in mature adipocytes, alternative approaches might be required. Several chemical and peptide-based PRMT inhibitors are available, though the specificity and cellular activity of the current PRMT inhibitors remains to be improved (1,65). It would be amenable to treat cultured adipocytes with the inhibitors to see whether inhibition of PRMTs causes any molecular and phenotypic changes such as gene expression, cytokine secretion, and lipid accumulation. Additionally creation of inducible knockout models for individual PRMTs would be ideal tools for studying the specific roles of PRMTs in adipose tissues. The effect of tissue-specific knockout might be observed in a normal

physiological condition or under a pathological condition such as diet-induced obesity.

4.2 Biological roles and enzymatic functions of PRMT7

Though PRMT7 is not required for the adipocyte differentiation and cell proliferation of C3H10T1/2 cells, it has significant roles in development and survival of organisms. PRMT7 orthologs were found in eukaryotes including *Mus musculus, Xenopus laevis, Drosophila melanogaster, C. elegans* and *Arabiddopsis thaliana*, but not in yeasts or in prokaryotes (22). Loss of PRMT7 ortholog *Dart7* in *D. melanogaster* leads to pupal lethality (255). PRMT7 knockout mice die within 5-10 days after birth (33). The causes of lethality are not characterized. Defective B-cell development and humoral immunity were identified in mice with a B-cell specific knockout of PRMT7. However, these mice seem to live much longer as compared to the whole body PRMT7 knockout mice (5 to 10 months or longer vs. 5 to 10 days) (33). Therefore, additional roles of PRMT7 might exist *in vivo*. Indeed, a study suggests that PRMT7 might be required for cellular pluripotency, because the expression of PRMT7 decreases as embryonic stem cells and embryonic germ cells differentiate (216). Another study showed that PRMT7 represses neuronal gene expression and differentiation (87). PRMT7 also participate in the cellular responses to DNA damage (31). Therefore, future studies are necessary to connect the context-dependent functions of PRMT7 to its systematic role in organisms.

The type of methylation that PRMT7 catalyzes remains controversial. Although many studies have linked PRMT7 to symmetrical dimethylation on substrates *in vivo* (28,30-33,87,128), the *in vitro* characterization of PRMT7 activity is contradictory. The Pestka group showed that the recombinant human PRMT7 is capable of forming symmetrical dimethylation on the common PRMT substrates including histones, myelin basic protein, and Sm proteins (26). The methylation products were analyzed by thin-layer chromatography (TLC), demonstrating a type II activity of PRMT7. The resolution of TLC-based assay and the possible contamination with PRMT5 during immunoprecipitation procedure make these results questionable (23). Although the purified FLAG-PRMT7 was not associated with the ectopically expressed HA-PRMT5, the potential interaction of FLAG-PRMT7 with the endogenous PRMT5 cannot be ruled out, because PRMT5 is known to interact with the anti-FLAG M2 antibody (256). Additionally, the lack of a negative control involving a PRMT7 catalytic mutant in the methyltransferase activity assay makes it difficult to conclude that the methylation on the substrates resulted only from the PRMT7 activity.

In contrast, Clarke and colleagues determined that recombinant human and mouse PRMT7 only catalyze monomethylation *in vitro* by high-resolution cation exchange chromatography (22-25). Their results are consistent with the characterization of PRMT7 homologue in *Trypanosoma Brucei* (257). Crystallographic analyses also indicated that *T. Brucei* PRMT7 (TbPRMT7) and *C. elegans* PRMT7 (cePRMT7) contains a narrow arginine-binding pocket, which can

only accommodate unmethylated arginine as a substrate for methylation (35,258). The mouse PRMT7 (mPRMT7) may bind monomethylarginine (40). However, mPRMT7 only catalyzes monomethylation *in vitro* (24). In addition, PRMT7 targets a RXR sequence within lysine and arginine-rich regions instead of the common motifs for other PRMTs (24,25). These findings suggest the unique substrate specificity and type III activity of PRMT7. In this regard, both type I and type II PRMTs might not be able to compensate the function of PRMT7.

More biochemical and structural characterization is required to fully understand PRMT7's catalytic activity and substrate specificity. In addition, the connection between PRMT7 and cellular levels of MMA are not established. MMA was identified on a large set of proteins involved in RNA processing and transcriptional regulation (8,9). Transcriptional inhibition using Actinomycin D alters the levels of MMA without changes in ADMA and SDMA on the substrates, indicating MMA represent a distinct biological mark rather than an intermediate of dimethylation (9). Thus, it would be interesting to determine whether these MMA marks are catalyzed by PRMT7 and the functional consequence of monomethylation on substrates.

4.3 Adipogenic function of JMJD6 and beyond

The investigation of JMJD6 leads to the conclusion that JMJD6 is required for adipocyte differentiation. Surprisingly, the data suggests that the participation of JMJD6 in the differentiation is independent of its enzymatic activity. This finding

and the previous report on JMJD6 function in splicing (159) suggest that JMJD6 may instead acts as a scaffold protein for both transcription and RNA splicing. The differences between enzymatic and scaffold functions in gene regulation might be examined by transcriptome analysis on the knockout and the enzymatic dead knock-in models for JMJD6. Moreover, since JMJD6 interacts with the multiple proteins involved in transcriptional regulation and RNA processing (142,151,159,229), it is likely that JMJD6 recruits its binding partners to the chromatin to facilitate these processes. This hypothesis can be examined by ChIP assays for factor binding at *Ppar*γ and *Cebp*α gene loci.

The chromatin association of JMJD6 is mediated by BRD4 and that is crucial for the expression of the *Ppar*γ and *Cebp*α genes during adipocyte differentiation. The results are consistent with the previous study showing BRD4 and JMJD6 cooperates to regulate gene expression in HEK293T and HeLa cells (142). However, the previous study connected the transcriptional function of JMJD6 to its dual demethylase activities targeting both histone H4R3 and 7SK RNA, which is inconsistent with my results showing that the enzymatic activity is not essential. Moreover, unlike their results, neither overexpression nor knockdown of JMJD6 affected the symmetrical dimethylation of histone H4R3 (**Figure A2**). Therefore, further research is required to understand the mechanisms by which JMJD6 facilitates gene expression in the context of adipogenesis. JMJD6 directly interacts with CDK9 (142), a kinase component of P-TEFb complex. One possible scenario is that JMJD6-BRD4 complex competes

with HEXIM1/7SK snRNP complex for CDK9 binding independently of JMJD6's enzymatic activity. HEXIM1/7SK snRNP is a general transcription inhibitor that sequesters P-TEFb in an inactive complex (259). The displacement of HEXIM1/7SK snRNP from CDK9 leads to upregulation of P-TEFb activity to promote RNA polymerase II elongation. The functional interplay between JMJD6, transcriptional regulators, and RNA processing factors would be an interesting topic for future research.

Another emerging question from the present research is how JMJD6 is involved in the post-transcriptional regulation of C/EBPβ and C/EBPδ. One might hypothesize that JMJD6 affects the formation and nuclear export of the mRNPs. JMJD6 interacts with U2AF65 and SR proteins (151,159,229). These proteins are not just splicing factors but also the adaptors for recruiting the transcription/export complex (TREX) and export receptor NXF1/TAP to mRNAs (260-262). The data showed that Aly/REF, a component of TREX complex, and the export receptor NXF1/TAP immunoprecipitates with the Flag-tagged JMJD6 from the NIH3T3 cell extracts (**Figure A3**). This suggests that JMJD6 associates with the conserved RNA export machinery. Further studies are required to examine whether depletion of JMJD6 can cause a general defect in RNA export or whether a defect only occurs on specific RNAs. The biological significance of the interaction between JMJD6 and RNA export proteins also need to be determined.

Although two distinct enzymatic functions were assigned to JMJD6, the data presented in this thesis suggests that JMJD6 has an additional role independent of its enzymatic activity. JMJD6 regulates gene expression via multiple mechanisms, and that is likely to link transcription to RNA processing and translation. The role of JMJD6 in cellular differentiation is clearly not limited to adipocytes, because the JMJD6 knockout mice exhibit defective differentiation in multiple tissues (154-156,263). In addition to its role in differentiation, JMJD6 regulates cell proliferation. Knockdown of JMJD6 slowed proliferation of C3H10T1/2 and NIH3T3 cells (**Figure A4**), which is consistent with other studies using different cell types (157,264,265). Therefore, JMJD6 is a critical gene expression regulator for both cellular differentiation and proliferation, which might explain why the deletion of JMJD6 causes wide spread defects in mice. Finally, the enzymatic activity of JMJD6 remains a puzzling question in the field. More efforts are required to clarify the enzyme activities and the biological relevance of JMJD6 catalytic functions.

Appendices

Table A1 List of primers

Primers for cloning	Sequence 5' to 3'
shCtrl forward	GATCCTGGCGGCGAGTGAAGTACGTGATAAGT GTGCTGTCCTTATCACGTACTTCACTCGCCGCC ATTTTTGGAAA
shCtrl reverse	AGCTTTTCCAAAAATGGCGGCGAGTGAAGTAC GTGATAAGGACAGCACACTTATCACGTACTTC ACTCGCCGCCAGG
shGFP forward	GATCCCGAAGCAGCACGACTTCTTCGTGTGCTG TCCGAAGTCGTGCTGCTTCTTTTTGGAAA
shGFP reverse	AGCTTTTCCAAAAAGAAGCAGCACGACTTCTT CGGACAGCACACGAAGAAGTCGTGCTGCTTCGG
shJ6-2 forward	GATCCCCGATGAACCACAAGAGCAAGTGTGCT GTCCTTGCTCTTGTGGTTCATCGTTTTTGGAAA
shJ6-2 reverse	AGCTTTTCCAAAAACGATGAACCACAAGAGCA AGGACAGCACACTTGCTCTTGTGGTTCATCGGG
shJ6-3 forward	GATCCCGCTGACACCCAGAGAACAAGTGTGCTG TCCTTGTTCTCTGGGTGTCAGCTTTTTGGAAA
shJ6-3 reverse	AGCTTTTCCAAAAAGCTGACACCCAGAGAACAA GGACAGCACACTTGTTCTCTGGGTGTCAGCGG
pBABE-JMJD6 forward	CCCGGATCCCTCACGATGAACCACAAGAGCAAG
pBABE-JMJD6 reverse	CGGTCGACTTATCATTTGTCATCGTCGTCCTTGT AGTCCCTGGAGGAGCTGCGCTC
pcDNA3.1-JMJD6 forward	same as pBABE-JMJD6
pcDNA3.1-JMJD6 reverse	CGAAGCTTTTATCATTTGTCATCGTCGTCCTTGTA GTCCCTGGAGGAGCTGCGCTC
pcDNA3.1-HIF1AN forward	CCCGAATTCGTAGAGATGGCGGCGACGGCAGCC
pcDNA3.1-HIF1AN reverse	CGAAGCTTTTATTATTTGTCATCGTCGTCCTTGT AGTCGTTGTAACGGCCTTTAAT
JMJD6 H187AD189A forward	GCGTTCTGGAACTGGGATTGCCATCGCCCCTCT GGGG
JMJD6 H187AD189A reverse	CCCCAGAGGGGCGATGGCAATCCCAGTTCCAGA ACGC
GST-JMJD6 2-414 forward	GCGGATCCAACCACAAGAGCAAGAAGCGCATC
GST-JMJD6 2-414 reverse	CCCAAGCTTTTATTTGTCATCGTCGTCCTTGTAG TCCCTGGAGGAGCTGCGCTCTTTGCT

Primers for qPCR	**Sequence 5' to 3'**
Jmjd6 forward	GAGCGCCTCAAAAGGAAATA
Jmjd6 reverse	TGGGCACCTTGTAGTCTTCC
Pparγ2 forward	GCATGGTGCCTTCGCTGATGC
Pparγ2 reverse	AGGCCTGTTGTAGAGCTGGGT
Cebpα foward	CCGGCCGCCTTCAACGAC
Cebpα reverse	CTCCTCGCGGGGCTCTTGTTT
Cebpβ forward	TGGACAAGCTGAGCGACGAG
Cebpβ reverse	TGTGCTGCGTCTCCAGGTTG
Cebpδ forward	TTCAGCGCCTACATTGACTC
Cebpδ reverse	TGTGGTTGCTGTTGAAGAGG
Srebp1c forward	AGCTGTCGGGGTAGCGTCTG
Srebp1c reverse	GAGAGTTGGCACCTGGGCTG
Fasn forward	CGTGTTGGCCTACACCCAGAGCT
Fasn reverse	GGCAGCAGGGCCTCCAGCACCTT
Fabp4 forward	GCGTGGAATTCGATGAAATCA
Fabp4 reverse	CCCGCCATCTAGGGTTATGA
AdipoQ forward	CAGTGGATCTGACGACACCA
AdipoQ reverse	CGAATGGGTACATTGGGAAC
5S rRNA forward	GTCTACGGACATACCACCCTG
5S rRNA reverse	TACAGCACCCGGTATTCCCAG
Primers for ChIP	**Sequence 5' to 3'**
Pparγ2 -9.5kb forward	TTCTTCCCAGTAGGAACTGCAT
Pparγ2 -9.5kb reverse	GATCACTCAGTTGGCATTTCTC
Pparγ2 -0.3kb forward	TGGCCAAATACGTTTATCTGG
Pparγ2 -0.3kb reverse	CCAGTGACCCACACATTCACTG
Pparγ2 TSS forward	ATTCCCACCTCTCCCAAATA
Pparγ2 TSS reverse	GCTCTGGGTCAACAGGAGAA
Pparγ2 +20kb forward	CGAGTCTGTGGGGATAAAGC
Pparγ2 +20kb reverse	CCAAAACAACTCCCCACAAC
Pparγ2 +96kb forward	CTTGGGAGCTACAGCCTTGTG
Pparγ2 +96kb reverse	GCTGTGGTGAAACGACAGTTATTA
Pparγ2 +100kb forward	CCGGGCTCCCTAGATGTAGAC
Pparγ2 +100kb reverse	ACCCTTTCTGAGCGACAACCT
Cebpα -0.3kb forward	CTCCCTAGTGTTGGCTGGAA
Cebpα -0.3kb reverse	GGTGAGTGGGGAGCATAGTG
Cebpα TSS forward	GCCCGACCCTCTATAAAAGC
Cebpα TSS reverse	GGCTCCACCTCGTAGAAGTC
Cebpα +2.2kb forward	GCAGTGTGCACGTCTATGCT
Cebpα +2.2kb reverse	AGCCCACTTCATTTCATTGG
Cebpα +8kb forward	CACAGTGTCTGCTCGCTGTT
Cebpα +8kb reverse	ATTACAGTGCCGCCAGGTAG

Cebpα +37kb forward	CCCCAATCTTCCCTCAAATGA
Cebpα +37kb reverse	GGAGCCCGGAACCAGAA
Cebpβ -0.4kb *forward*	CGTGTAGCTGGAGGAACGAT
Cebpβ -0.4kb *reverse*	CTCGGGAACACGGAGGAG
Cebpδ -0.1kb *forward*	AGGAGGGAAGGCAAGGAGT
Cebpδ -0.1kb *reverse*	CTTTTCTAGCCCCAGCTGAC

Figure A1.

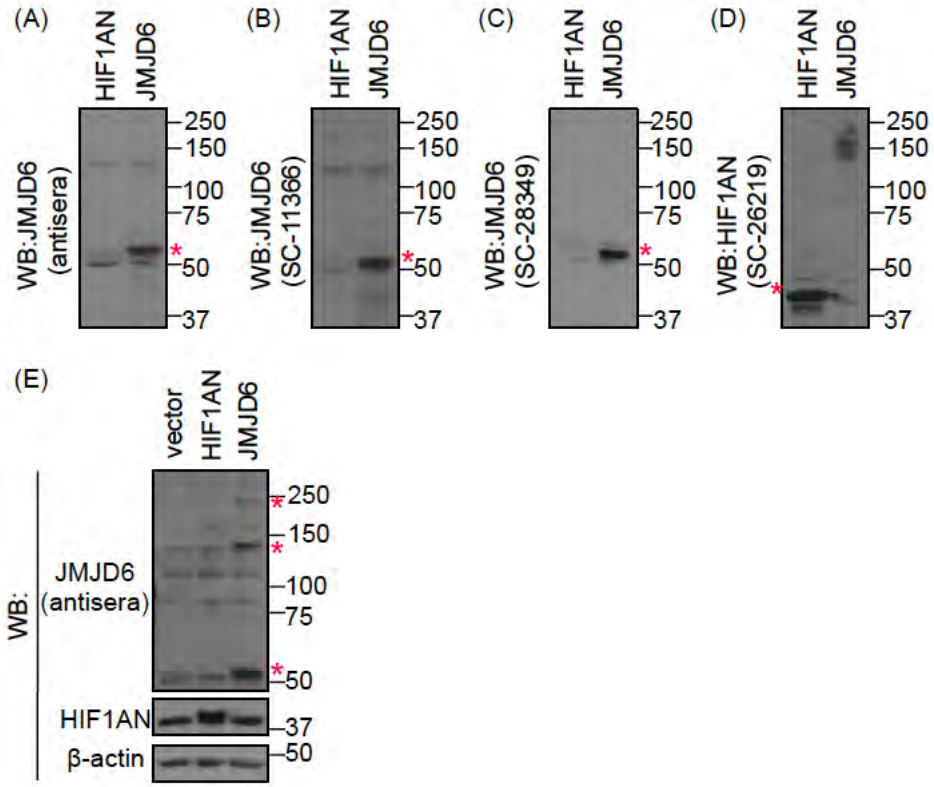

Figure A1. Evaluation of the specificity of JMJD6 antisera and antibodies. Representative western blots probed with **(A)** the JMJD6 antisera, **(B and C)** the commercial antibodies for JMJD6, and **(D)** the commercial antibody for HIF1AN, a different JmjC protein. JMJD6 and HIF1AN proteins were *in vitro* translated from 1 µg of the pcDNA3.1(-) plasmids encoding either FLAG-tagged mouse JMJD6 or HIF1AN using a TNT Quick coupled Transcription/Translation Systems (Promega) according to the manufacturer's instructions. 15 µl of the in vitro translated mixture was run on 10% SDS-PAGE and Western blotting was performed. **(E)** Representative western blots for JMJD6 and HIF1AN expression in the 24 h post-transfected C3H10T1/2 cells. 2 µg of the pcDNA3.1(-) plasmids encoding either FLAG-tagged mouse JMJD6 or HIF1AN, or the empty vector were transiently transfected into C3H10T1/2 cells using Lipofectamine 2000 according to the manufacturer's instructions. 20 µg of the total cell lysate was run on 10% SDS-PAGE for electrophoresis and Western blotting. β-ACTIN was probed as a loading control. Red asterisks indicate the bands corresponding to the target monomer and oligomeric proteins.

Figure A2.

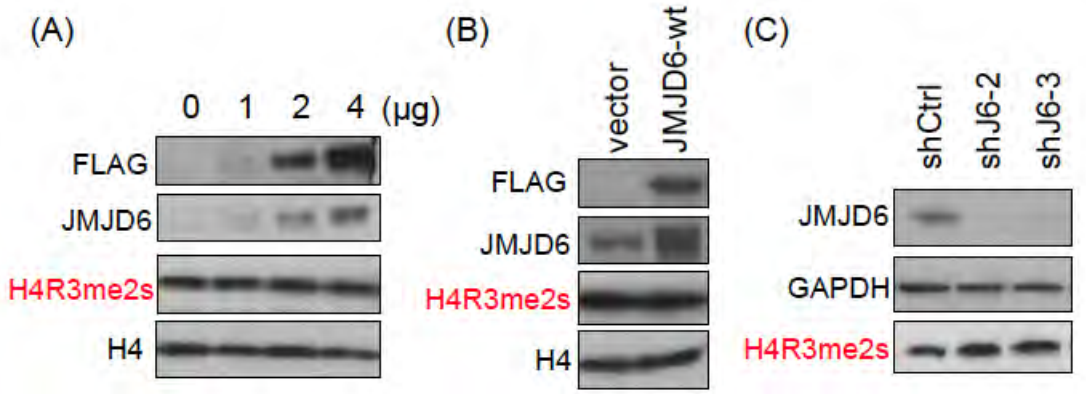

Figure A2. Over-expression and knockdown of JMJD6 has no effect on symmetrical dimethylation of histone H4R3.
(A) NIH3T3 cells were transfected with the pcDNA3.1 plasmid encoding Flag-tagged wild type JMJD6 **(B)** The pBABE empty vector and the construct encoding FLAG-tagged wild type JMJD6 (JMJD6-wt) were introduced into NIH3T3 cells by retroviral infection. **(C)** The scramble control (shCtrl) construct and the lentiviral shRNA constructs (shJ6-2 and shJ6-3) were introduced into NIH3T3 cells by lentiviral infection. The protein extracts were analyzed by western blot with the antibodies as indicated. (n=1)

Figure A3.

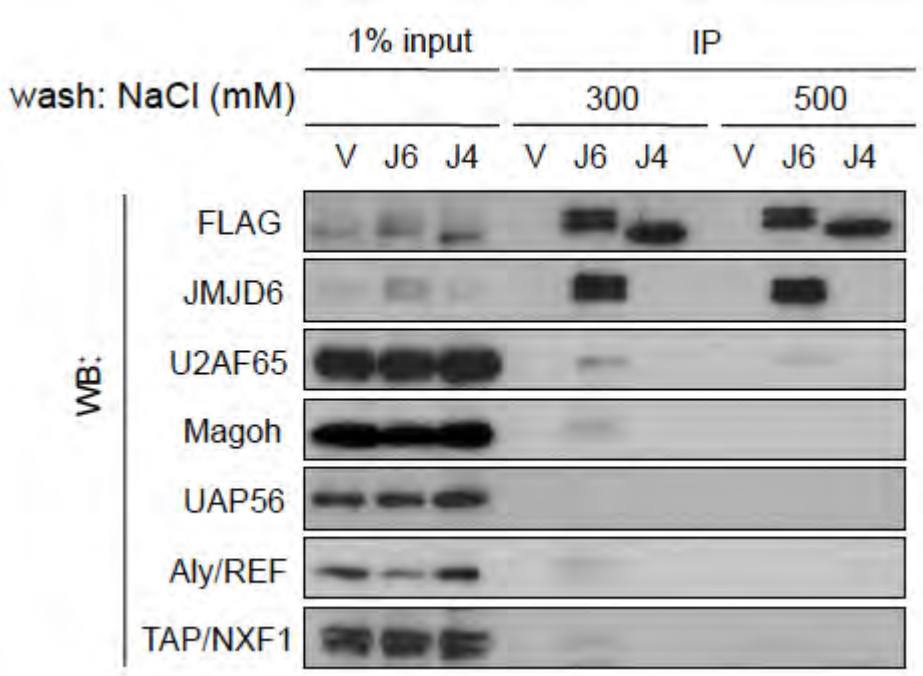

Figure A3. JMJD6 interacts with the proteins involved in RNA splicing and nuclear export.
The pBABE empty vector, the construct encoding FLAG-tagged JMJD6 (J6), and the construct encoding FLAG-tagged JMJD4 (J4) were individually introduced into NIH3T3 cells by retroviral infection. The protein extracts were subjected to immunoprecipitation using M2-beads (Sigma). After washing with the buffer containing either 300 mM or 500 mM NaCl, the interacting proteins were eluded and analyzed by western blot. The blots were probed with the antibodies as indicated. (n=1)

Figure A4.

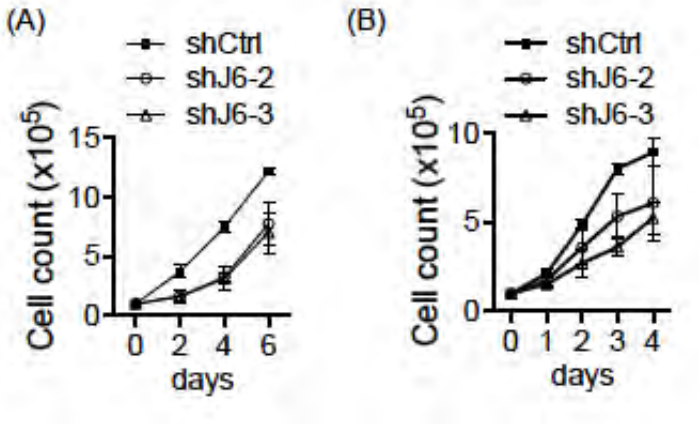

Figure A4. Knockdown of JMJD6 slows cell proliferation.
The scramble control (shCtrl) construct and the lentiviral shRNA constructs (shJ6-2 and shJ6-3) were introduced into **(A)** NIH3T3 and **(B)** C3H10T1/2 cells by lentiviral infection. $1x10^5$ cells were seeded in 6-well plates. The cells were counted at the day as indicated. (SEM, n=3)

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